Agricultural Research Service

National Program 108
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CONTENTS

2.10

CRIS Title: Prevention of Losses from Colibacillosis and O157:H7 in Cattle and Swine
CRIS: 3625-32420-001
Scientists: Casey TA, Dean-Nystrom EA, Sharma VK
Location: Pre-Harvest Food Safety and Enteric Diseases Research Unit, NADC, Ames, IA
Contact: 515-663-7728 (P); 515-663-7458 (F); tcasey@nadc.ars.usda.gov

2.14

CRIS Title: Microbial Factors-Pathogenesis of Sub-Acute Acidosis (SARA) in Cattle to Assure Food Safety
CRIS: 3625-32000-041
Scientists: Rasmussen M, Goff JP, Horst RL
Location: Periparture Diseases of Cattle Unit, NADC, Ames, IA
Contact: 515-663-7350 (P); 515-663-7669 (F); mrasmuss@nadc.ars.usda.gov

Section 3 Pre-harvest Swine

3.1

CRIS Title: Microbial Competitive Exclusion to Reduce Epizootic Pathogenic Bacteria in Swine and Cattle
CRIS: 6202-42000-010
Scientists: Anderson RC, Beier RC, Callaway TR, Harvey RB, Hume ME, Nisbet DJ
Location: Food and Feed Safety Research Unit; SPARC, College Station, TX
Contact: 979-260-9317 (P); 979-260-9332 (F); anderson@ffsru.tamu.edu

3.5

CRIS Title: Salmonella-Host Interactions
CRIS: 3625-42000-006
Scientists: Stabel TJ
Location: Pre-Harvest Food Safety and Enteric Diseases Research Unit, NADC, Ames, IA
Contact: 515-663-7292 (P); 515-663-7458 (F); tstabel@nadc.ars.usda.gov

3.8

CRIS Title: Ecology and Epidemiology of *Salmonella* and Other Foodborne Pathogens in Livestock, Primarily Swine
CRIS: 3625-42000-005
Scientists: Wesley IV, Hurd HS, Ziemer CJ
Location: Pre-Harvest Food Safety and Enteric Diseases Research Unit, NADC, Ames, IA
Contact: 515-663-7291 (P); 515-663-7458 (F); iwesley@nadc.ars.usda.gov

Section 4 Pre-harvest Poultry

4.1

CRIS Title: Immunomodulation of the Avian Innate Immune Response to Prevent Bacterial Infections in Poultry
CRIS: 6202-42000-008
Scientists: Kogut MH, Crippen TL, He H
Location: Food and Feed Safety Unit, SPARC, College Station, TX
Contact: 979-260-3772 (P); 979-260-9332 (F); kogut@ffsru.tamu.edu

4.4	**CRIS Title:**	Prevention and Control of *Salmonella* and Other Enteropathogens in Poultry During Growout
	CRIS:	6202-42000-011
	Scientists:	Kubena LF, Byrd JA, Ziprin RL, Hume ME
	Location:	Food and Feed Safety Unit, SPARC, College Station, TX
	Contact:	979-260-9249 (P); 979-260-9332 (F); Kubena@ffsru.tamu.edu
4.8	**CRIS Title:**	Control of Salmonella in Domestic Animals
	CRIS:	6612-42000-019
	Scientists:	Bailey JS, Cox NA, Craven SE, Stern NJ
	Location:	Poultry Microbiological Safety Research Unit, Athens, GA
	Contact:	706-546-3356 (P); 706-546-3771 (F); jsbailey@ars.usda.gov
4.12	**CRIS Title:**	*Campylobacter* spp. Epidemiology, Methods Development and Interventions in Poultry.
	CRIS:	6612-42000-031
	Scientists:	Stern NJ, Cox NA, Line JE, Hiett KL, Siragusa G
	Location:	Poultry Microbiological Safety Research Unit, Athens, GA
	Contact:	706-546-3516 (P); 706-546-3771 (F); nstern@ars.usda.gov
4.16	**CRIS Title:**	Pathogenesis, Detection, and Control of *Salmonella enteritidis* and Other Salmonellae in Chickens
	CRIS:	6612-32000-017
	Scientists:	Gast RK, Petter JG, Holt PS, Mitchell BW, Swayne DE
	Location:	Southeast Poultry Research Laboratory, Athens, GA
	Contact:	706-546-3445 (P); 706-546-3161 (F); rgast@seprl.usda.gov
4.20	**CRIS Title:**	Epidemiology and Ecology of *Salmonella Enteritidis* in Commercial Poultry Flocks
	CRIS:	6612-42000-022
	Scientists:	Petter JG, Holt PS, Gast RK, Swayne DS, Mitchell BW
	Location:	Southeast Poultry Research Laboratory, Athens, GA
	Contact:	706-546-3446 (P); 706-546-3161 (F); jpetter@seprl.usda.gov
4.22	**CRIS Title:**	Disease Related Problems of Poultry Production and Processing
	CRIS:	6226-42000-005
	Scientists:	Huff GR, Huff WE, Donoghue AM
	Location:	Poultry Production Product Safety Research Unit, Fayetteville, AR
	Contacts:	501-575-2413 (P); 501-575-4202 (F); donoghue@mail.uark.edu

Section 5 Detection Methods

5.1	**CRIS Title:**	Biosensor Processes for Detecting Pathogenic Bacteria in Foods
	CRIS:	1935-42000-040
	Scientists:	Shu-I Tu, Irwin P, Yu L, Brewster J
	Location:	Microbial Biophysics Biochemistry, ERRC, Wyndmoor, PA
	Contact:	215-233-6466 (P); 215-233-6581 (F); stu@arserrc.gov

5.6	**CRIS Title:**	New Technology and Systems to Detect and Prevent Chemical and Microbial Food Contaminants
	CRIS:	1935-42000-035
	Scientists:	Shu-I Tu, Linton R
	Location:	Purdue University and Eastern Regional Research Center, Wyndmoor, PA
	Contact:	215-233-6466 (P); 215-233-6581 (F); stu@arserrc.gov
5.10	**CRIS Title:**	Development of Methods and Strategies to Improve the Microbiological Safety of Aquaculture Products
	CRIS:	1935-42000-036
	Scientists:	Richards GP, Kingsley D
	Location:	Microbial Food Safety Research Unit, Delaware State University, Dover, DE
	Contact:	302-857-9419 (P); 302-857-6451 (F); grichard@dsc.edu
5.14	**CRIS Title:**	Identification and Application of Novel Technologies for Detection of Pathogenic Microorganisms in Foods and Environmental Samples
	CRIS:	5325-42000-027
	Scientists:	Stanker LH, Brandon DL, Gaffield W, Wong R, Binder RG, Haddon W
	Location:	Food Safety and Health Research Unit, WWRC, Albany, CA
	Contact:	510-559-5984 (P); 510-559-6162 (F); lstanker@pw.usda.gov
5.18	**CRIS Title:**	Molecular Systematics and Diagnostics for Parasites of Food and food Animals.
	CRIS:	1265-42000-002
	Scientists:	Rosenthal BM, Hoberg EP
	Location:	Biosystematics Unit, PBESL, ANRI, BARC (formerly BNPCU), Beltsville, MD
	Contact:	301-504-5408 (P), 301-504-8979 (F); brosenth@anri.barc.usda.gov
5.21	**CRIS Title:**	Molecular Genomics of Plant Pathogens and Food Safety Microorganisms
	CRIS:	3620-42000-022
	Scientists:	Kurtzman CP, Labeda DL, Nakamura, LK, Peterson SW, O'Donnell K, (vacant)
	Location:	Microbial Properties Research Unit, NCAUR, Peoria, IL
	Contact:	309-681-6561 (P); 309-681-6672 (F); kurtzman@mail.ncaur.usda.gov

Section 6 Post-harvest Cattle and Swine

6.1	**CRIS Title:**	Control of Pathogenic and Spoilage Bacteria on Red Meat
	CRIS:	5438-32000-014
	Scientists:	Berry ED, Rivera-Betancourt M, Koohmaraie, M.
	Location:	Meats Research Unit, U.S. MARC, Clay Center, NE
	Contact:	Ph. 402-762-4225; Fax 402-762-4149; berry@email.marc.usda.gov Ph. 402-762-4221; Fax 402-762-4149; Koohmaraie@email.marc.usda.gov
6.5	**CRIS Title:**	Development of On-line Verification and Intervention Procedures for HACCP in Slaughter/Processing Systems
	CRIS:	5438-42000-002
	Scientists:	Barkocy Gallagher GA, Koohmaraie M, vacant (2).
	Location:	Meats Research Unit, MARC, Clay Center, NE
	Contact:	402-762-4228 (P); 402-762-4149 (F); gallagher@email.marc.usda.gov 402-762-4221 (p); 402-762-4149 (F); Koohmaraie@email.marc.usda.gov

6.8 **CRIS Title:** Quantitative Determination of Pathogen Reduction During Animal Slaughter and Food Processing
CRIS: 1935-42000-034
Scientists: Luchansky JB, vacancy (vice Palumbo S), Medina M, Feder I
Location: Microbial Food Safety Research Unit, ERRC, Wyndmoor, PA
Contact: 215-233-6620 (P); 215-233-6568 (F); jluchansky@arserrc.gov

Section 7 Post-harvest Poultry

7.1 **CRIS Title:** Development of Technology for Automated On-Line Inspection of Animal Carcasses /Produce
CRIS: 1265-42000-007
Scientists: Chen YR, Chao K, Kim MS, Lefcourt A, Delwiche SR
Location: Instrumentation and Sensing Laboratory, BARC, Beltsville, MD
Contact: 301-504-8450; Fax:301-504-9466; cheny@ba.ars.usda.gov

7.6 **CRIS Title:** Development of Imaging Technology for the Automated On-Line Inspection of Poultry Products
CRIS: 6612-41420-030
Scientists: Windham WR, Lawrence KC, Smith DP, Lyon CE
Location: Poultry Processing and Meat Quality Unit, RRRC, Athens, GA
Contact: 706-546-3515 (P); 706-546-3633 (F); rwindham@saa.ars.usda.gov

7.8 **CRIS Title:** Engineering Innovations and Micro Developments to Reduce Contamination on Poultry Processing Equipment and Poultry
CRIS: 6612-41420-003
Scientists: Dickens JA, Buhr RJ, Cason JA, Hinton Jr. A , Lyon CE
Location: Poultry Processing and Meat Quality Unit, RRRC, Athens, GA
Contact: 706-546-3205 (P); 706-546-3633 (F); adickens@saa.ars.usda.gov

7.13 **CRIS Title:** Reduction & Control of Pathogens Associated with Food Processing Surfaces
CRIS: 6612-41420-006
Scientist: Arnold JW , Lyon CE
Location: Poultry Processing and Meat Quality Unit, RRRC, Athens, GA
Contact: 706-546-3515 (P); 706-546-3633 (F); jarnold@saa.ars.usda.gov

7.17 **CRIS Title:** Microbial Ecology and Transmission of Human Pathogens during Poultry Processing
CRIS: 6612-41420-007
Scientists: Meinersmann RJ, Berrang ME, Lyon CE
Location: Poultry Processing and Meat Quality Unit, RRC, Athens, GA
Contact: 706-546-3236 (P); 706-546-3633 (F); rmeiners@saa.ars.usda.gov

7.20 **CRIS Title:** Effects of Processing Treatments on Safety & Quality of Raw and Cooked Poultry Products
CRIS: 6612-41420-005
Scientists: Lyon CE, Jones DR, Young LL
Location: Poultry Processing and Meat Quality Unit, RRRC, Athens, GA
Contact: 706-546-3418 (P); 706-546-3633 (F); glyon@saa.ars.usda.gov

7.23

CRIS Title: Adhesion and Control of Human Pathogens to and on Surfaces (Part A: Poultry)
CRIS: 5325-42000-022
Scientists: Mandrell RE, Charkowski AO, Cooley MB, Friedman M, Gorski L, Kint S, Miller WG
Location: Food Safety and Health Unit, WRRC, Albany, CA.
Contact: 510-559-5610 (P); 510-559-6162 (F); mandrell@pw.usda.gov

7.26

CRIS Title: Advanced Technologies for Reduction of Microorganisms and Particulate Matter in Food Processing
CRIS: 5325-42000-024
Scientists: Tsai L-S, Hernlem B, Huxsoll C, Robertson G
Location: Process and Engineering Unit, WRRC, Albany, CA
Contact: 510-559-5878 (P); 510-559-5862 (F); lstsai@pw.usda.gov

Section 8 Post-harvest Processing Interventions

8.1

CRIS Title: Improve Microbiological Safety & Shelf-Life of Food by Treatment with Ionizing Radiation
CRIS: 1935-42000-033
Scientists: Thayer DW, Fan X, Niemira BA, Rajkowski KT, Sommers CH.
Location: Food Safety Research Unit, ERRC, Wyndmoor, PA
Contact: 215-233-6582 (P); 215-233-6406 (F); dthayer@arserrc.gov

8.7

CRIS Title: Assurance of Microbiological Safety of Thermally Processed Foods.
CRIS: 1935-42000-028
Scientists: Juneja VK, Novak J, Huang L
Location: Food Safety Research Unit, ERRC, Wyndmoor, PA
Contact: 215-233-6500 (P); 213-233-6406 (F); vjuneja@arserrc.gov

8.11

CRIS Title: Development of Intervention Processes to Enhance the Microbiological Safety of Heat Sensitive Foods
CRIS: 1935-41420-004
Scientists: Kozempel MF, Geveke DJ, Craig JC, Goldberg N, McAloon AJ
Location: Engineering Science Research Unit, ERRC, Wyndmoor, PA
Contact: 215-233-6588 (P); 215-233-6795 (F); mkozempel@arserrc.gov

8.14

CRIS Title: New Technologies to Improve and Assess Food Safety in Muscle Foods
CRIS: 1265-41420-002
Scientists: Nedoluha PC, Berry BW, Solomon MB, Spanier AM
Location: Food Technology and Safety Laboratory, BARC, Beltsville, MD
Contact: 301-504-8400 (P); 301-504-8438 (F); msolomon@anri.barc.usda.gov

Section 9 Virulence and Risk Assessment

9.1

CRIS Title: Stress Responses and Virulence Expression of Bacterial Pathogens in Food Environments
CRIS: 1935-42000-031
Scientists: Fratamico PM, Bhaduri S, Bayles DO, Solow BT
Location: Microbial Food Safety Research Unit, ERRC, Wyndmoor, PA
Contact: 215-233-6525 (P): 215-233-6581 (F): pfratamico@arserrc.gov

9.7

CRIS Title: Post-Harvest Predictive Microbiology and Process Risk Assessment
CRIS: 1935-42000-041-00 (from merge of 1935-42000-032-00 and1935-42000-029-00)
Scientists: Tamplin ML, Oscar TP, Zaika LL
Location: Microbial Food Safety Research Unit, ERRC, Wyndmoor, PA
Contact: 215-836-3794 (tel); 215-233-6581 (fax); mtamplin@arserrc.gov

Section 10 Produce and Animal Manure

10.1

CRIS Title: New Technologies for Decontamination of Fresh Fruits and Vegetables Containing Human Pathogens
CRIS: 1935-41420-003
Scientists: Sapers GM, Annous BA, Sites J., Fett WF, Goldberg NM, Hicks, KB
Contact: 215-233-6417 (P); 215-233-6406 (F); gsapers@arserrc.gov

10.5

CRIS Title: New Chemical, Physical, and Biological Technologies for Decontaminating Sprouting Seed and Produce with Easily Damageable Surfaces
CRIS: 1935-41420-005
Scientists: Fett WF, Liao C-H., Ukuku D, Sapers GM, Hicks KB
Location: Plant Science and Technology Research Unit, ERRC, Wyndmoor, PA
Contact: 215-233-6418 (P), 215-233-6406 (F); wfett@arserrc.gov

10.8

CRIS Title: Decontamination of Alfalfa Seeds and Sprouts with Ozone
CRIS: 1935-41420-005
Scientists: Fett WF
Location: Plant Science & Technology Research Unit, ERRC, Wyndmoor, PA
Contact: 215-233-6418 (P); 215-233-6406 (F); wfett@arserrc.gov

10.9

CRIS Title: Microbial Safety of Fresh Fruits and Vegetables
CRIS: 1275-42000-002
Scientists: Bhagwat AA, Conway WS, McEvoy J, Wachtel M, Vacant (Food Technologist)
Location: Produce Quality and Safety Laboratory, PSI, BARC, Beltsville, Maryland
Contact: 301-504-5106 (P): 301-504-5107 (F); bhagwata@ba.ars.usda.gov

10.11

CRIS Title: Adhesion and Control of Human Pathogens to and on Surfaces (Part B: Produce)
CRIS: 5325-42000-022
Scientists: Mandrell RE, Charkowski AO, Cooley MB, Friedman M, Gorski L, Kint S, Miller WG
Location: Food Safety and Health Unit, WRRC, Albany, CA.
Contact: 510-559-5610 (P); 510-559-6162 (F); mandrell@pw.usda.gov

10.14
CRIS Title: Treatment of Animal Manure to Prevent Pathogen Transmission
CRIS: 5325-42000-023
Scientists: Ravva SV, Duffy, B, Mandrell RE.
Location: Food Safety and Health Research Unit, WRRC, Albany, CA
Contact: 510-559-6176 (P); 510-559-6162 (F); subba@pw.usda.gov

10.15
CRIS Title: Prevent Zoonotic Pathogen Transmission and Improve the Value of dairy Manure to the Environment
CRIS: 1265-31420-001
Scientists: Millner PD, Karnes JS, (vacant)
Location: Animal Waste Pathogen Laboratory, ANRI, BARC, Beltsville, MD
Contact: 301-504-8163 (P); 301-504-8370 (F); pmillner@asrr.arsusda.gov

10.17
CRIS Title: Reducing Pathogen Incidence and Contamination Potential of Spent Poultry Litters
CRIS: 6612-42000-027
Scientists: Line JE, Siragusa G, Hiett K, Stern NJ
Location: Poultry Microbiological Safety Research Unit, Athens, GA
Contact: 706-546-3522 (P); 706-546-3771 (F); eline@ars.usda.gov

Section 11 Toxic Chemicals and Drug Residues

11.1
CRIS Title: Advanced Techniques for the Analysis of Chemical Residues in Foods
CRIS: 1935-42000-039
Scientists: Lehotay SJ, Schneider MJ, Pensabene JW, Fiddler W
Location: Food Safety Research Unit, ERRC, Wyndmoor, PA
Contact: 215-233-6433 (P); 215-233-6642 (F); slehotay@arserrc.gov

11.5
CRIS Title: Alternate Solvent Systems and Techniques for Food Analysis
CRIS: 3620-42520-001
Scientists: King JW.
Location: New Crops Research, NCAUR, Peoria, Il
Contact: 309-681-6203 (P); kingjw@mail.ncaur.usda.gov

11.8
CRIS Title: Dioxins and Other Environmental Contaminants in Food.
CRIS: 5442-42000-002
Scientists: Huwe JK, Shappell NW, Shelver WL, Hakk H, Garber EAE, Larsen GL, Smith DJ
Location: Animal Metabolism-Agricultural Chemicals Research Unit, RRVARC, Fargo, ND
Contact: 701-239-1288 (P); 701-239-1430 (F); huwej@fargo.ars.usda.gov

11.11
CRIS Title: Absorption, Distribution, Metabolism & Elimination of Veterinary Drugs and Mycotoxins in Food Animals
CRIS: 5442-32000-007
Scientists: Smith DJ, Huwe JK, Shappell NW, Hakk H, Garber EAE, Larsen GL, Shelver WL.
Location: Animal Metabolism-Agricultural Chemicals Research Unit, RRVARC, Fargo, ND
Contact: 701-239-1238 (P); 701-239-1430 (F); smithd@fargo.ars.usda.gov

Section 12 Mycotoxins

12.1

CRIS Title: Control of Fusarium Mycotoxins and Diseases in Corn and Small Grains
CRIS: 3620-42000-018
Scientists: Plattner RD, Desjardins AE, Muhitch MJ, Proctor RH
Location: Mycotoxin Research Unit, NCAUR, Peoria, IL
Contact: 309-681-6579 (P); 309-681-6689 (F); plattnrd@mail.ncaur.usda.gov

12.4

CRIS Title: Control of *Fusarium graminearum* Mycotoxins in Wheat, Barley, and Corn
CRIS: 3620-42000-021
Scientists: Desjardins AE, Alexander NJ, McCormick SP, Proctor RH, Plattner RD
Location: Mycotoxin Research Unit, NCAUR, Peoria, IL
Contact: 309-681-6379 (P); 309-681-6689 (F); desjarae@mail.ncaur.usda.gov

12.6

CRIS Title: Detection, Identification, and Surveillance of Mycotoxins in Cereals and Other Foods
CRIS: 3620-42000-023
Scientists: Maragos CM, Dombrink-Kurtzman MA, Plattner R.D, Vesonder RF
Location: Mycotoxin Research Unit, NCAUR, Peoria, IL
Contact: 309-681-6266 (P); 309-681-6267 (F); maragocm@mail.ncaur.usda.gov

12.8

CRIS Title: Critical Control Points in Corn Resistance/Susceptibility to *Aspergillus flavus* and Aflatoxin
CRIS: 3620-42000-020
Scientists: Wicklow DT, Gardner HW
Location: Mycotoxin Research Unit, NCAUR, Peoria, IL
Contact: 309-681-6243 (P); 309-681-6689 (F); wicklodt@mail.ncaur.usda.gov

12.13

CRIS Title: Biochemical, Physical, Microbiological Management for Control of Mycotoxin Contamination of Peanuts
CRIS: 6604-42000-006
Scientists: Dorner JW, Horn BW
Location: National Peanut Research Laboratory, Dawson, GA
Contact: 229-995-7400 (P); 229-995-7416 (F); jdorner@nprl.usda.gov

12.16

CRIS Title: Aflatoxin Control Through Targeting Gene Cluster Governing Aflatoxin Synthesis in Corn and Cottonseed
CRIS: 6435-41420-002
Scientists: Bhatnagar D, Cleveland TE, Cary JW, Yu J, Chang PK, Ehrlich KC, Klich MA, Rajasekaran K, Brown RL
Location: Food and Feed Safety Unit, SRRC, New Orleans, LA
Contact: 504-286-4388 (P): 504-286-4269 (F); dbhatnag@srrc.ars.udsa.gov

12.21

CRIS Title: Aflatoxin Control Through Addition of Enhancement of Antifungal Genes in Corn and Cotton.
CRIS: 6435-42000-012
Scientists: Cleveland TE, Cary JW, Rajasekaran K, Brown RL, Klich MA, Jacks TJ, Yu J
Location: Food and Feed Safety Unit, SRRC, New Orleans, LA
Contact: 504-286-4387 (P): 504-286-4269 (F); eclevela@srrc.ars.udsa.gov

12.26	**CRIS Title:**	Modification of Fungal Community Structure to Improve Food Safety
	CRIS:	6435-42000-014
	Scientists:	Cotty PJ, Mellon JE
	Location:	Food and Feed Safety Unit, SRRC, New Orleans, LA
	Contact:	504-286-4391 (P): 504-286-4419 (F); pjcotty@srrc.ars.udsa.gov
12.31	**CRIS Title:**	Control of Toxic Endophytic Fungi of Corn and Grasses
	CRIS:	6612-42000-021
	Scientists:	Porter JK, Yates ID, Norred WP, Riley RT, Bacon CW, (vacant)
	Location:	Toxicology and Mycotoxin Research, RRRC, Athens, GA
	Contact:	706-546-3158 (P); 706-546-3116 (F); cbacon@saa.ars
12.34	**CRIS Title:**	Reduction of Fusarium Mycotoxins as Concerns in Agricultural Commodities
	CRIS:	6612-42000-020
	Scientists:	Voss KA, Porter JK, Bacon CW, Riley RT, Norred WP
	Location:	Toxicology and Mycotoxin Research, RRRC, Athens, GA
	Contact:	706-546-3158 (P); 706-546-3116 (F); cbacon@saa.ars.usda.gov
12.38	**CRIS Title:**	Agronomic, Environmental, and Resistant Germplasm Effects on Aflatoxins and other Mycotoxins
	CRIS:	6402-42000-001
	Scientists:	Abbas HK, Meredith WR, Bruns HA
	Location:	Crop Genetics Production Research, Stoneville, MS
	Contact:	662-686-5313 (P);662-686-5218 (F); habbas@ars.usda.gov
12.41	**CRIS Title:**	The Use of Aflatoxigenic Strains of *Aspergillus Flavus* to Prevent Aflatoxin Contamination
	CRIS:	5344-42000-012
	Scientists:	Henneberry T
	Location:	Western Cotton Research Laboratory, Phoenix, Arizona
	Contact:	602-437-0121 (P);602-437-1274 (F); thenneberry@wcrl.ars.usda.gov
12.43	**CRIS Title:**	Reduction of Aflatoxin in Tree Nuts Through Control of Insect Pests Using Natural Products
	CRIS:	5325-42000-031
	Scientists:	Campbell BC, Light DM, Roitman JN, Buttery RG, Wong R
	Location:	Plant Protection Research Unit, WRRC, Albany, CA
	Contact:	510-559-5846 (P); 510-559-5777 (F); bcc@pw.usda.gov
12.45	**CRIS Title:**	Inhibition of Tree Nut Contamination by Aflatoxins and Related Mycotoxins using Natural Products
	CRIS:	5325-42000-032
	Scientists:	Campbell BC, Molyneux RJ, Bayman P, Hua SST
	Location:	Plant Protection Research Unit, WRRC, Albany, CA
	Contact:	510-559-5846 (P); 510-559-5777 (F); bcc@pw.usda.gov
12.48	**CRIS Title:**	Removal of Aflatoxin Contamination from Human Foods in Real Time by Imaging Techniques
	CRIS:	5325-42000-030
	Scientists:	Schatzki, TF, Keagy PM, Pearson TC
	Location:	Cereal Product Utilization Research Unit, WRRC, Albany, CA
	Contact:	510-559-5672 (P); 510-559-5777 (F)

Section 13 Toxic Plants and Chemicals in Water and Environment

13.1

CRIS Title: Poisoning of Livestock by Various Larkspur (*Delphinium*) Species
CRIS: 5428-32630-008
Scientists: Pfister JA, Gardner DR, Ralphs MH, Stegelmeier BL, Panter K, James LF, Lee S
Location: Poisonous Plant Research Laboratory, NPA, Logan, UT
Contact: 435-752-2941 (P); 435-753-5681 (F); lfjpprl@cc.usu.edu

13.5

CRIS Title: *Astragalus* and *Oxytropis* Poisoning in Livestock
CRIS: 5428-32000-008
Scientists: James L, Ralphs M, Stegelmeier B, Panter K, Pfister J, Gardner D, Lee S
Location: Poisonous Plant Research Laboratory, Logan, UT
Contact: 435-752-2941 (p); 435/753-5681 (F); lfjpprl@cc.usu.edu

13.9

CRIS Title: Livestock Poisoning by Pyrrolizidine Alkaloids and Other Hepatotoxic and Teratogenic Plants
CRIS: 5428-32000-009
Scientists: Gardner DR, Stegelmeier BL, James LF, Panter KE, Pfister JA, Lee S
Location: Poisonous Plant Research Laboratory, Logan, UT
Contact: 435-752-2941 (P); 435-753-5681 (F); lfjpprl@cc.usu.edu

13.12

CRIS Title: *Pinus* and *Gutierrezia* Species: Toxicoses and Abortion in Livestock
CRIS: 5428-31320-002
Scientists: Panter KE, James LF, Gardner DR, Pfister JA, Ralphs MA, Stegelmeier B, Lee S.
Location: Poisonous Plant Research Laboratory, NPA, Logan, UT
Contact: 435-752-2941 (P); 435-753-5681 (F); lfjpprl@cc.usu.edu

13.16

CRIS Title: Prevent the Occurrence of Toxins in Water to Protect Food and the Environment.
CRIS: 5442-42000-003
Scientists: Larsen GL, Garber EAE, Hakk H, Shelver WL, Shappell NW
Location: Animal Metabolism-Agricultural Chemicals Research Unit, RRVARC, Fargo, ND
Contact: 701-239-1231 (P); 701-239-1430 (F); larseng@fargo.ars.usda.gov

13.20

CRIS Title: The Effect of Plant Genetics and Zinc on Cadmium Concentration and Bioavailability in Crops
CRIS: 1265-42000-005
Scientists: Chaney RL
Location: Animal Manure and By-Products Laboratory, ANRI, BARC, Beltsville, MD
Contact: 301-504-8324 (P); 301-504-5048 (F); rchaney@asrr.arsusda.gov

13.23

CRIS Title: Identify Mechanisms of Isoflavonoid Induction in Legumes and their Phytoestrogenic Effects
CRIS: 6435-42000-013
Scientists: Cleveland TE, Boue SM, Ehrlich KC
Location: Food and Feed Safety Unit, SRRC, New Orleans, LA
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EXECUTIVE SUMMARY

This report summarizes ARS research progress in National Program 108 - Food Safety. The research efforts are now categorized into 13 areas reflecting the Food Action Plan. Key Research findings from National Program 108 during the past year follow. Additional information on the Food Safety Program may be obtained at http://nps.ars.usda.gov/programs/programs.htm?NPNUMBER108

Transmission of Campylobacter through birds

The most important source of Campylobacter in broiler production has been controversial. Effective interventions for Camylobacter in poultry are lacking because we have not identified effective control measures nor where to apply them. ARS scientists (Athens GA) isolated and genetically characterized Campylobacter from commercial breeder flocks and their offspring broiler flocks. Using sensitive genetic techniques they demonstrated that Campylobacter could be transmitted through bird generations via the fertile hatching egg. This new information points the way to devising effective controls that will break the cycle of infection in broiler chicks and control a serious human health hazard.

Preharvest Certification of Pork Protects Consumers

Consumers want the confidence that Trichinella is no longer a human health risk associated with eating pork. ARS scientists together with the pork industry have developed a certification system based on knowledge of risk factors, detection methods and good management practices . Certification requires pork producers to meet certain management criteria that eliminate risk of exposure of pigs to the Trichinella parasite. This certification program has been adopted by the pork industry, and APHIS and FSIS are currently developing regulations for management of the program. Certification should help USDA establish equivalency agreements with foreign markets regarding the safety of U.S. pork.

Rapid detection of viruses in aquaculture products

Rapid methods are needed for the detection of enteric viruses such as hepatitis A, Norwalk and rotavirus in food and water. ARS succeeded in developing a much safer and more rapid analytical method for the cell-culture-based enumeration of hepatitis A virus, and human and simian rotavirus using enhanced chemiluminescence technology. The new method reduces by five days the previous assay procedure, and eliminates the use of radioactive isotopes in the detection protocol. The new method will have broad-based appeal for regulatory and action agency monitoring of aquaculture products such as oysters; and will be particularly useful in determining the effectiveness of processing strategies for the inactivation of viruses.

Levels of E. coli in beef

ARS determined the relationship between cattle contamination and subsequent carcass contamination was evaluated. Results showed an unexpectedly high numbers of animals per lot entered the slaughter plant carrying *E. coli* O157:H7/NM; however, very few carcasses were still contaminated after processing. These data have contributed to the food safety and policy debates

regarding the commonness of *E. coli* O157:H7/NM, the usefulness of sampling procedures, and strategies to eliminate *E. coli* 0157:H7/NM contamination of the beef supply.

Ecology of *Campylobacter* in processing plants
ARS research to evaluate and trace the distribution of *Campylobacter jejuni* in poultry processing plants suggests that the bacterial pathogen exists in two types; one that readily shares DNA among other members of the species; the another where no exchange occurs. The types that do not readily share DNA were not associated with chicken samples, however, they did include most of the strains associated with Guillian-Barre Syndrome, a progressively debilitating neurological disorder in humans.

Cooling of retail foods
Inadequate cooling of foods in retail food operations may allow the bacterium *Clostridium perfringens* to grow to potentially hazardous infective dose levels. ARS has now established the safe cooling rate for cured beef, pork, chicken and uncured chicken by defining the time and temperature needed to ensure safety in relation to control of *C. pefringens*. A predictive model has been developed to predict growth from spores at temperatures applicable to the cooling of cooked meats. The information will enable regulatory agencies and the food industry to evaluate the safety of cooked products.

Irradiation treatment to inactivate pathogens on hot-dogs
ARS evaluated the use of irradiation to eliminate the bacterial pathogen *Listeria monocytogenes* from hot-dogs. Studies concluded that a 99.999% (5-log) reduction of this pathogen was achieved with a radiation dose of 3.6kGy, thus meeting the regulatory goal of the Food and Drug Administration for this pathogen. Differences in radiation sensitivity were discovered that depended on product formulation, thus, the irradiation processing step would require product dependent adjustment.

Intervention strategies for ground meats
In the normal processes of breaking down the animal carcass into smaller meat cuts and trim, there are additional opportunities to spread or increase bacterial contamination. ARS designed a combination treatment process for the microbiological decontamination of pork trim prior to grinding. The processes were shown to reduce and control populations of fecal bacteria on pork trim and in the resultant ground pork. The work provides industry with a processes to improve both the microbiological safety and shelf life of ground pork products, and will assist processors in meeting the proposed *Salmonella* performance standards for fresh pork sausages.

Assuring the safety of apple cider
There is a critical need to assure the safety of fresh, unpasteurized fruit juices such as apple cider. Removal of pathogens from the surface of fruit before processing is considered a critical control point in the processing of apple juice. Previous ARS studies had demonstrated limitations in the efficiency of washing apples as a means of reducing microbial populations, even when fruit was washed with 5% hydrogen peroxide. ARS has now demonstrated the conditions for improving the

efficiency of hydrogen peroxide treatments by mechanical detachment of adhering bacteria, and by improving contact of the attached bacteria and wash solution. This improvement brings the Food and Drug Administrations 99.999% (5-log) populations reduction target for unpasteurized apple cider within reach.

Decontamination of sprouts

Naturally contaminated seeds must be decontaminated before being used to grow sprouts for human consumption. ARS had previously shown that laboratory contaminated seeds could be decontaminated with 20,000 ppm of free chlorine providing a 99.99% (4-log) reduction in pathogens. ARS has now demonstrated that this method was effective in sanitizing naturally contaminated seed. As a result sprout growers, consumers and regulatory agencies can have a greater confidence in the safety of sprouts grown from seeds treated in this manner.

Tissue promotor can target aflatoxin prevention genes in corn

Safe reliable effective means are needed to prevent mycotoxins, in particular aflatoxin, produced by certain fungi from contaminating crops and entering the food supply. ARS scientists (Peoria IL) have developed a technological tool, a gene promoter with the ability to express genes added to corn plants in a precise, tissue specific manner in kernel pedicels. Pedicels are a common site for infection in corn plants by mycotoxin producing fungi. When this tissue-specific promoter is refined and made user-friendly it will enable corn breeders to target novel genes only to the pedicel, thus increasing the safety of the genetic change made to prevent fungal infection and aflatoxin production in the edible corn kernel.

More sensitive monoclonal antibodies to detect DON in food products

Commercial assays for deoxynivalenol (DON) have insufficient sensitivity and are inadequate for screening for this mycotoxin in human food. ARS scientists (Peoria IL) produced three monoclonal antibodies against DON and using these antibodies developed enzyme linked immunosorbent assays (ELISAs). The assays which are the most sensitive ELISAs yet reported for DON, were tested with wheat samples, and a CRADA is being negotiated with a mycotoxin kit manufacturer. This rapid assay technology will assist plant breeders in identifying plant resistance in order to reduce the occurrence of these toxins in the field. It will also enable both producers and the regulatory agencies to screen commodities more economically, rapidly and accurately for the presence of the toxin.

Aflatoxin found in natural desert habitats

To successfully and regularly prevent aflatoxin in food commodities we need to to identify ecological niches harboring reservoirs of *A flavus*. ARS scientists (New Orleans) examined natural habitats of the Sonoran desert and found that key food and shelter sources for wildlife supported high population of *A. flavus;* legume fruits in particular, were found frequently contaminated with aflatoxins. This is the first evidence that contamination is a frequent, natural phenomenon that may affect animals even in habitats not directly involved in growing food crops. Thus reservoirs of *A flavus* not previously recognized exist in natural habitats and must be considered by aflatoxin management programs.

CRIS Title: Antimicrobial Resistance Research
CRIS: 6612-42000-028
Scientists: Cray PJF, Englen MD, Gray JT, Hudson CR
Location: Antimicrobial Resistance Research Unit, RRRC, Athens, GA
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Summary Project Aims:
Use of antimicrobials has increased in both human and veterinary medicine. This is due in part to the availability of the antimicrobials and the efficacy they impart in control of certain infectious diseases. However, use of antimicrobials can lead to the development of resistance to the antimicrobials. Antimicrobial resistance (AR) can either diminish effectiveness or render an antimicrobial ineffective as a therapeutic. Although use may result in a portion of bacteria that are resistant, the exact fate of this population in terms of persistence and transmission has been difficult to determine. Use patterns in veterinary medicine (therapeutic versus subtherapeutic use) also complicates the picture. Additionally, while transmission of resistant bacteria from animals to humans occurs, it has been difficult to assess the extent to which this occurs and the impact transmission has on actual dissemination of resistant populations. Despite growing concerns, information regarding the development and spread of AR in food borne and commensal bacteria is limited, and its impact on human health is poorly defined. Our goal is to identify and determine the etiologic fraction that may be attributed to the use of antimicrobials in animal production which impacts human health. This program leverages the information obtained through our participation in the National Antimicrobial Resistance Monitoring System-Enteric Bacteria (NARMS; 6612-42000-028-02R) to complete the following CRIS objectives: 1) Identify the genetic mechanisms associated with the development and maintenance of AR and develop rapid tests to identify resistance genes; 2) Determine the *in vivo* (particularly in poultry) and *in vitro* effect the acquisition of resistance confers on the bacterium; and 3) Conduct prospective ecological studies to better define the acquisition, transmission, and dissemination of AR including, as a component, participation in the NARMS.

Summary Accomplishments During Entire Project:
This is a new CRIS project and the Research Leader and three SYs were hired in 2000. Accomplishments are documented in subordinate CRIS projects (6612-42000-028-01T and 6612-42000-028-02R). This is the first complete year of the CRIS dedicated to antimicrobial resistance research. During 998 a rapid test to identify/confirm *Salmonella typhimurium* DT104 was developed. DT104 is a multiple resistant *S. typhimurium* which has been associated with an increase in morbidity and mortality in both humans and animals. In 1999, we challenged broiler chicks on the day of hatch with either a sensitive or penta-resistant *S. typhimurium* DT104 to assess the effect on virulence and/or colonization in chickens. We determined that penta-resistant bacteria do not cause clinical illness in broiler chicks; however, we did observe a significant increase in the numbers of birds that were colonized in the penta-resistant group. These data indicate that acquisition of multiple resistance does affect colonization rates and may impact the numbers of bacteria that may reach the food chain. Additionally, we have optimized methods for determination of resistance in *Campylobacter* spp. which reduces preparation time, minimizes variability and can be easily

implemented into existing testing assays employing microbroth techniques. For the NARMS program, the number of isolates tested has doubled each year over the past three years. Molecular tests have also been developed and are in place to rapidly speciate *Campylobacter*. Sentinel test site submissions (laboratories which submit diagnostic samples that represent different geographic locations in the US) have continued to expand which add to the power of the data. Through these efforts, the animal industry has recognized the importance of the AR problem and they have developed and implemented prudent antimicrobial drug use guidelines.

Summary 2000 Accomplishments:
Antimicrobial test methodologies for *Campylobacter* are technically difficult, costly and often difficult to compare to agar dilution which is considered the 'gold standard'. A microbroth dilution assay has been developed which is cost effective, comparable to existing methodologies, easier than the agar dilution, and compatible with current equipment. This work was funded through a CRADA with the Animal Health Institute and will be presented to the National Committee for Clinical Laboratory Standards (NCCLS) for adoption as a recommended testing methodology. NCCLS determines the most accurate means of antimicrobial susceptibility testing and disseminates this information worldwide. Additionally, through a collaborative project with Dr. David Reeves at the University of Georgia, we have determined that antimicrobial use patterns on swine operations do affect the development of resistance. However, for *Salmonella* species, results appear to be serotype dependent which confounds both interpretation of results and design/implementation of mitigation strategies. Interestingly, use patterns do not appear to have the same effect on the development of resistance in *Campylobacter* species. Analysis of *Enterococci* and generic *E. coli* isolates is ongoing. There is a lack of information concerning antimicrobial resistance patterns in food borne pathogens of animal origin. Together with the FDA-CVM, USDA-APHIS, and USDA-FSIS, we determined antimicrobial resistance patterns in *Salmonella* and *Campylobacter* isolates of animal origin. Over 8,500 *Salmonella* isolates and 750 *Campylobacter* isolates were tested against 17 antimicrobials for *Salmonella* and eight antimicrobials for *Campylobacter*, and a 320 page report was generated. Testing for *Enterococci* and generic *E. coli* also commenced. Results from this program will facilitate the identification of resistance in humans and animals as it arises, provide descriptive data on the extent and temporal trends of antimicrobial susceptibility, provide timely information to veterinarians and physicians, prolong the lifespan of approved drugs by promoting the prudent and judicious use of antimicrobials, and identify areas for more detailed investigation.

Projected Research Accomplishments During Next 3 Years:
Expected accomplishments over the next several years include an increased understanding of basic mechanisms of resistance, epidemiology of resistance under field conditions, transfer of resistance between bacterial species (particularly zoonotic and commensal bacteria), development of a more accurate assessment of the human health impact from agricultural use of antimicrobials, testing and implementation of mitigation procedures, and rapid test development. During 2001, in addition to further expansion of the unit, we will develop knowledge of the epidemiology of antimicrobial resistance and associated risk factors that may contribute to resistance development/spread/ transmission in broiler operations. Basic mechanisms of resistance will be explored and the role of gene transfer between food borne pathogens and commensal bacteria will begin along with

investigations on the organization of the antibiotic resistance genes in DT104 and the mechanisms by which these genes are acquired. In 2002 we will continue study of basic mechanisms of resistance and develop rapid tests to identify resistance attributes. In 2003 we will begin study of resistance attributes/patterns which have emerged over the previous years. Participation in NARMS will also continue throughout the period.

Technology Transfer:
The technologies which have been developed in this and subordinate CRIS projects have been transferred to the FDA-CVM for use in policy decisions, to FSIS, to USDA-APHIS for use in field studies, to USDA-ARS for use in policy decisions and to develop CRIS objectives and other ancillary research projects, to the GAO for use in discussing the issue with Congress, to pharmaceutical industries for use in developing drug use guideline and product development, to diagnostic laboratories (both human and veterinary), to commodity groups for educational purposes and to CDC, veterinary organizations and commodity groups for use in developing prudent use guidelines. We anticipate that this transfer will continue over the lifetime of the project. The results are available through written and electronic media formats.

PUBLICATIONS:

Tollefson, L., Angulo, F.J., Fedorka-Cray, P.J.. National surveillance for antibiotic resistance in zoonotic enteric pathogens. 1999. Veterinary Clinics of North America. p.141-150.

Davies, P.R., Turkson, P.K., Funk, J.A., Nichols, M.A., Ladely, S.R., Fedorka-Cray, P.J. Comparison of methods for isolating salmonella bacteria from feces of naturally infected pigs. 2000. J. Appl. Microbiol. v.89 p.169-177.

Fedorka-Cray, P.J., Gray, J.T., Wray, C. *Salmonella* infections in pigs. *Salmonella* in Domestic Animals. C. Wray and A. Wray, eds. 2000. CABI International, London, p.191-208.

Kabagambe, E.K., Wells, S.J., Garber, L.P., Salman, M.D., Wagner, B., Fedorka-Cray, P.J. Risk factors for fecal shedding of *Salmonella* in 91 US dairy herds in 1996. 1999. Prevent. Vet. Med. v.413 p.177-194.

PROCEEDINGS/ABSTRACTS:

Bahnson, P., Fedorka-Cray, P.J.. 1999. The association of antimicrobial resistance patterns and reported usage of antimicrobial in commercial growing pig production. Intl. Symp. Epidemiol. Ctrl. Salmonella in Pork. Abstract p.240-241.

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Bailey, J.S., Stern, N.J., Fedorka-Cray, P.J., Craven, S.E., Cox, N.A. A multi-state epidemiological investigation of sources and movement of *Salmonella* through integrated poultry operations. Proceedings of United States Animal Health Association. 1999. Abstract p.194-198.

Cox, N.A., Stern, N.J., Musgrove, M.T., Bailey, J.S., Craven, S.E., Fedorka-Cray, P.J. Prevalence and level of *Campylobacter* in commercial broiler breeders and broilers. 2000. Poultry Science 79 (Suppl. 101), Abstract p.5.

Craven, S.E., Stern, N.J., Bailey, J.S., Cox, N.A., Fedorka-Cray, P.J. Ribotyping for the differentiation of *Clostridium perfringens* isolates from poultry production and processing. 2000. Poultry Sci. v.79(S). Abstract p.75

Dargatz, D.A., Fedorka-Cray, P.J., Petersen, K.E., Hollinger, K., Wineland, N.E., Tollefson, L., Ferris, K. Prevalence and distribution of antimicrobial resistance. American Association of Bovine Practitioners Proceedings. 1999. Abstract

Fedorka-Cray, P.J., Stern, N.J., Muhmed, W.A. Historical analysis of antimicrobial resistance in *Campylobacter*. 1999. Poultry Science. v.78(S). Abstract p.84.

Fedorka-Cray, P.J., Dargatz, D.A., Wineland, N.E., Miller, M.A., Tollefson, L., Hollinger, K., Petersen, K.E., Ferris, K. Antimicrobial susceptibility monitoring. 30th Annual Meeting of the American Association of Swine Practitioners, St. Louis, MO. Judicious Antimicrobial Use. 1999. Abstract p.9-10.

Fedorka-Cray, P.J., Dargatz, D.A., Wineland, N.E., Miller, M.A., Tollefson, L., Petersen, K.E. Antimicrobial susceptibility patterns of *Salmonella* isolates. USAHA 1999. Abstract p.1.

Fedorka-Cray, P.J., Miller, M.A., Dargatz, D.A., Wineland, N.E., Tollefson, L. Prevalence/Trends of quinolone resistance in *Salmonella* isolates from animals in the USA. World Health Organization Technical Report. 1999. p.1-4.

Gray, J.T. Fedorka-Cray, P.J. Serological detection of swine exposed to *Salmonella* spp. Annual meeting of USAHA/AAVLD, Microbiology Session B. San Diego, CA Oct. 8th 1999. Abstract.

Hollinger, K., Silvers, L., Fedorka-Cray, P.J., Angulo, F., Tollefson, L., Stamey, K. Antibiotic resistance in *Salmonella* enterica serotypes Heidelberg, Kentucky and Thompson isolated from human and broiler chicken sources. 39th Interscience Conference on Antimicrobial Agents & Chemotherapy. 1999. Abstract p.101

Ladely, S.R., Fedorka-Cray, P.J., Bailey, J.S., Stern, N.J. Colonization of broiler chicks by *Salmonella typhimurium* DT104. Poultry Sci. 1999. v.84 (Suppl. 1). Abstract p.103.

Traub-Dargatz, J.L., Lindsey, G.P., Fedorka-Cray, P.J., Ladely, S.R., Ferris, K. NAHMS-Equine '98 Study *Salmonella* spp. Fecal shedding in the U.S. horse population and presence of *Salmonella* Spp. in grain sources on equine operations 1998-1999. 9th International Society Veterinary Epidemiology and Economics. Abstract p.1034-1037

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CRIS Title: Antimicrobial Resistance to Enteric Bacteria in Food Producing Animals
CRIS: 6202-42000-013
Scientists: Bischoff KM, Beier RC, Genovese KJ, Poole TL.
Location: Food and Feed Safety Research Unit, SPARC, College Station, TX
Contact: 979-260-9306 (P); 979-260-9332 (F); bischoff@ffsru.tamu.edu

Summary Project Aims:
The increase in antimicrobial resistance in human food pathogens may be correlated with agricultural usage of antibiotics. This project focuses on developing gut models to aid research on the spread of specific antibiotic resistance genes between normal flora and gut enteropathogens. We are using the knowledge gained from these models to predict rates and methods of antimicrobial resistance acquisition. The work also focuses on developing alternative methods for use in lieu of antimicrobials to enhance growth, and on the effects of commercial disinfectants on antimicrobial resistance.

Summary 2000 Accomplishments:
A fundamental understanding of the process of antimicrobial resistance is needed in order to prevent the spread of unwanted resistant genes among the gut microbial ecosystem. We assessed the diversity of antimicrobial resistance phenotypes and genotypes in enterotoxigenic *Escherichia coli* isolates from swine. Although chloramphenicol has been removed from use in food animals since 1985, we found that chloramphenicol resistance persists at significant levels. This information implies that drug removal may not be sufficient to combat the persistence of antibiotic resistance, and necessitates the development of alternative resistance management strategies. We also used continuous culture models of gut microflora to determine the effect of vancomycin on bacteria within the continuous culture model and within the gut of animals. We found that vancomycin resistant enterococci growth was prevented in the model by the bacterial consortium, and also in the gut of animals that had been provided the bacteria from the gut model. This information may provide a method of decreasing the spread of vancomycin resistance between gut bacteria, and prolong the efficacious life of this important human antibiotic.

Summary Accomplishments During Entire Project:
This is a new project started during 2000; therefore, the accomplishments during the life of the project are the same as stated under 2000 Accomplishments.

Projected Research Accomplishments During Next 3 Years:
Continue development of an *in vitro* model of gut microflora for studying emergence and transfer of antibiotic resistance genes. We will also assess the links between multiple antibiotic resistance genotypes and virulence, determine if the use of commercial disinfectants is creating an antimicrobial resistance problem, and develop alternatives to antimicrobials. Continue these studies as well as determine if new alternatives are efficacious in controlling gut colonization by antimicrobial resistant enteropathogens. Sstudy the effect of dose and duration of several antibiotics on the rate of resistance acquisition by normal gut flora both in our *in vitro* gut models and in live animals.

Technology Transfer:
Although this is a new project started in 2000, we have already presented some of our research findings from our gut model work at various scientific meetings, have published one manuscript in a peer reviewed scientific journal, and submitted another. Managing antibiotic resistance is an inherently difficult problem, but we anticipate that practical technology arising from this work will reach the end user within 4-7 years.

PUBLICATIONS:

Beier, R.C., Oyofo, B.A., Spates, G.E. Occurrence of the toxin dehydroabietic acid in Salmonella typhimurium. Toxicon. 2000. v. 38. p. 337-346.

1.8

CRIS Title: Antimicrobic Resistance of Enteric Bacteria
CRIS: 3625-42000-004
Scientists: Stanton TB, Carlson SA
Location: Pre-Harvest Food Safety and Enteric Diseases Research Unit, National Animal Disease Center, Ames, IA
Contact: 515-663-7495 (P); 515-663-7458 (F); tstanton@nadc.ars.usda.gov

Summary Projects Aims:
The emergence of antibiotic resistant bacterial pathogens is a serious threat to the treatment and control of human disease. Antibiotic resistance in *Salmonella* directly threatens food safety. There is growing public concern that the use of antimicrobials in livestock management contributes to the development of antibiotic resistance of human pathogens. There is insufficient understanding of the mechanisms of antimicrobial resistance gene transfer among intestinal bacteria of food animals, of routes and potential for transmission of antimicrobial resistance determinants between food animals and humans, of the persistence of resistant bacterial strains, and of the relationship between antibiotic resistance and increased virulence. Our overall goal is to provide knowledge and develop strategies useful to prevent the expansion and reduce the incidence of antibiotic resistance in bacteria. Short-term, specific goals are to: (A) Analyze, at the molecular level, the pathogenesis, epidemiology, and antibiotic resistance of *Salmonella typhimurium* DT104 and related pathogens obtained from cattle and swine; (B) Evaluate possible associations between expression of virulence determinants and antibiotic resistance in bacterial pathogens; and (C) Identify basic mechanisms and selective pressures involved in the evolution and transfer of antibiotic resistance genes in intestinal bacteria of swine and cattle.

Summary 2000 Accomplishments:
This is a new project. This year we set out to identify transferable genes encoding resistance to aminoglycosides in *Salmonella typhimurium* DT104. Bacterial strains were obtained from the National Veterinary Services Laboratories while the research was performed at NADC. We identified three predominate genes encoding resistance to kanamycin, apramycin, and gentamicin. The results support the hypothesis that antibiotic resistance in *Salmonella typhimurium* DT104 is spreading by clonal expansion. We set out to study the relationships between antibiotic exposure and virulence in *Salmonella*. Bacterial strains were obtained from the National Veterinary Services Laboratories while the research was performed at NADC. We found that, within a large group of multiresistant *Salmonella*, cellular invasion capabilities were uniform. The results demonstrate that the hypervirulent abilities of multiresistant *Salmonella* are not restricted to the ability to physically enter cells.

Summary Accomplishments During Entire Project:
This project was initiated January 15, 2000. We developed a rapid, sensitive and specific PCR-based test for detecting multiple antibiotic resistant *Salmonella typhimurium* DT104 (DT104). This test will provide the basis for rapid pre- and/or post-harvest detection of an important foodborne pathogen. The implementation of this test will reduce the time needed to detect DT104 from

24-48 hours to 8-12 hours. That is, potentially contaminated meat can be detected before leaving the slaughterhouse. Thus a safer food supply will be an outcome of this research. This system was recently combined with a similar test for E. coli O157:H7 so that both pathogens could be detected simultaneously.

Projected Research Accomplishments During Next 3 Years:
Identify a cytotoxin that is secreted by hypervirulent multiple antibiotic resistant *Salmonella*. Identify and compare tetracycline resistances of intestinal bacteria from swine fed different levels of that antibiotic. Identify the molecular basis for selective expression of the *Salmonella* cytotoxin and the epidemiologic characteristics of *Salmonella* that express the cytotoxin. Isolate and identify unique bacterial strains which reside in the swine intestinal tract and which are resistant to tetracycline. Develop a non-hydrolyzable antibody that can neutralize the cytotoxin. Characterize the tetracycline resistance genes of the swine intestinal bacteria and evaluate the transmissibility of those genes to human pathogenic and non-pathogenic bacteria.

Technology Transfer:
DNA-based tests will allow rapid detection of DT104 contaminants in food and the environment. This test has been transferred to an ARS scientist for development as a detection method for food and fecal *S. typhimurium* DT104 contamination. This assay will benefit producers, packing plants, regulatory agencies, and ultimately, the consumer. Information regarding the use of ineffective penicillin-like antibiotics will provide important insight for the proper use of antibiotics on the farm. Information on the invasive potential of multiresistant *S. typhimurium* DT104 has been lacking and these new scientific data will help build a sound basis for policy decisions and public education. Identification of novel mechanisms in *S. typhimurium* DT104 host interaction and pathogenesis will allow the development of more effective vaccines.

PUBLICATIONS:

Carlson, S. A., Ferris, K. E. Augmentation of antibiotic resistance in *Salmonella typhimurium* DT104 following exposure to penicillin derivatives. 2000. Vet. Microbiol. v. 73 (1). p. 25-35.

Frana, T. S., Carlson, S. A., Griffith, R. W. Relative distribution and conservation of genes encoding aminoglycoside-modifying enzymes in *Salmonella enterica* serotype Typhimurium phagetype DT104. Appl. Environ. Microbiol. v. 67 (1). Accepted 10/5/00.

CRIS Title: Prevention of Infections by Enterohemorrhagic *Escherichia coli* and Non-host Adapted *Salmonella* in Cattle
CRIS: 5438-42000-004
Scientists: Laegreid WW, Chitko-McKown CG, Bono JL
Location: Animal Health Research Unit, MARC, Clay Center, NE
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Summary Project Aims:

Foodborne disease is a major public health concern in the U.S. The gastrointestinal tracts of livestock serve as the source of some of the more important bacteria which cause foodborne diseases, such as *Escherichia coli* O157:H7 and *Salmonella typhimurium* DT104. Thus, understanding the epidemiology and ecology of livestock infection with such bacteria, as well as the interaction of bacteria with livestock and other host species, is required for rational design of control strategies in the preharvest portion of the production cycle.

Developing improved diagnostic methods for important foodborne bacterial species allows for very accurate studies of natural infections in commercial and experimental herds of cattle. Sources of infection, dynamics of infection and effects of external influences (such as management, environment, etc.) are identified as potential control points to reduce prevalence of these bacteria in livestock, thereby, improving the safety of the U.S. food supply.

Foodborne disease is one of the most important issues in public health and animal agriculture. Changes in the production, processing, distribution and consumption of food have contributed to the emergence of new pathogens and to the increasing importance of old ones. This has markedly influenced both the domestic and international markets for meat and related products in the U.S. Prevention and control strategies for foodborne pathogens must be designed and implemented at all levels of the production cycle, including the preharvest period, to reduce the risk to consumers and economic impacts on producers.

Determining how to reduce microbial pathogens in food products, throughout food operations from farm to fork, is the most urgent food safety problem today. A major goal of this program is to develop tests that are precise and rapid enough to detect contamination in all foods prior to their entering into commerce. Equally important is the development of effective, reliable, and cost-effective methods to control or eliminate pathogens in/on food producing animals throughout production and processing.

Using epidemiological surveillance, a better definition of the economic impact of livestock diseases can be gained to better understand the ecology of emerging diseases and natural transmission cycles. This is needed to develop control strategies to prevent disease. The emergence of a new disease or introduction of an exotic disease into the U.S. could rapidly escalate into an epidemic due to the lack of resistance in host animals, absence of vaccines or effective drugs and limited resources to effectively manage the spread of these pathogens.

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Summary Accomplishments during Entire Project:
This CRIS project is a replacement for the 5438-42000-003 project which was scheduled to terminate in July, 1999. There have been four major accomplishments over the life of this project. First, we have shown that there is a clear and significant relationship between infection of live cattle with *E. coli* O157:H7 and contamination of beef carcasses. This relationship underpins the entire concept of preharvest food safety. Second, we have developed an unsurpassed panel of monoclonal antibodies for the detection and characterization of enterohemorrhagic *E. coli* and *Salmonella*, including type, group and virotype-specific antibodies. Several of these antibodies have been formatted for diagnostic tests, including one test which is widely used in the U.S. meat industry. Third, we have described the epidemiology of infection of cattle with enterohemorrhagic *E. coli* and *Salmonella typhimurium* DT104 throughout the production cycle. These studies have shown the differences in progression of infection between these types of bacteria in cattle and have partially quantified the transmission between infected and naive cattle, important parameters for designing preharvest control methods. Fourth, we have evaluated several potential intervention strategies to reduce fecal shedding of *E. coli* O157:H7. Results of these experiments will reduce utilization of plausible, but ineffective, control strategies for *E. coli* O157:H7 and have indicated strategies worthy of further development.

Summary 2000 Accomplishments:
We completed a survey of *E. coli* O157:H7 infection in feedlot cattle presented for slaughter. The prevalence of infection was found to be significantly higher than previously reported, 28% of all animals tested. Additionally, it was found that infection was widespread, with 72% of slaughter groups testing positive for *E. coli* O157:H7. This is the first comprehensive study of *E. coli* O157:H7 prevalence in fed cattle at slaughter and is the first demonstration of a significant association between prevalence of infection in the live animal and carcass contamination. These results clearly show that control of foodborne pathogens such as *E. coli* O157:H7 in the preharvest phase of production will have significant effects on carcass contamination and public health.

The transmission parameters for *E. coli* O157:H7 in naturally infected herds of cattle were determined. It was found that the basic reproductive rate of *E. coli* O157:H7 infection in cattle, a measure of the number of new infections caused by each infected individual, was approximately the same as that for smallpox in humans. This number is important because it is used to estimate the percentage of the population which will have to be covered by an effective preventive or control strategy to eliminate infection from the herd. This result will provide a baseline against which to evaluate newly developed control and prevention strategies for *E. coli* O157:H7 in cattle.

Projected Research Accomplishments during Next 3 Years:
During 2001, we expect to further refine methods to trace the flow of *E. coli* O157:H7 and related bacteria in the production environment. Studies to identify immunologic correlates of bacterial clearance in cattle will be initiated. During 2001- 2002, we will continue and extend studies to identify immunologic correlates of clearance of *E. coli* O157:H7 from the gastrointestinal tract

of cattle. Host and bacterial genetic response to infection will be examined. During 2001-2003, herd sampling and epidemiologic studies of *E. coli* reservoirs and transmission within and between herds will be continued.

Technology Transfer:
Reduction of numbers of cattle infected with *E. coli* O157:H7 should significantly reduce the risk of contamination of the U.S. meat supply. Methods to reduce numbers of infected cattle should be evaluated in naturally infected animals under production conditions to minimize costs of instituting ineffective methods and to estimate unintended outcomes such as adverse health effects. These studies found that two plausible control strategies were either ineffective (high levels of pen sanitation) or associated with significant adverse health and economic effects. Furthermore, the inadvertent beneficial effect of transport was demonstrated. These results have been communicated to cattle producer groups directly (NCBA) and through the lay press (Beef, September 1999).

There has been great interest in this methodology among industry, research, academic, diagnostic and regulatory groups. Scientists from major meat packing companies, several universities, NVSL, NAHMS and other ARS researchers have been provided with the protocol and/or been trained in the use of this technique in our laboratories. Widespread use of culture techniques with greater sensitivity will significantly improve monitoring of infected livestock, evaluation of intervention strategies, and detection of environmental contamination. As the recent outbreak of enterohemorrhagic *E. coli* O111 in Texas demonstrated, serotypes other than O157:H7 represent threats to the U.S. food supply. Serotypes O26, O111 and O103, among others, are relatively more common elsewhere in the world and our studies have shown them to be present in U.S. cattle. Use of monoclonal antibodies to these O antigen types in rapid diagnostic and screening tests will provide and estimate of the potential risk to the U.S. meat supply. Constraints on the development of rational pre-harvest control strategies still remain, particularly incomplete knowledge of the ecology/epidemiology of the relevant bacteria in a production setting.

PUBLICATIONS:

Basaraba, R.J., Byerly, A.N., Stewart, G.C., Mosier, D.A., Fenwick, B.W., Chengappa, M.M., Laegreid, W.W. Actin enhances the haemolytic activity of *Escherichia coli*. 1998. Microbiology v. 144. p. 1845-1852.

Elder, R. O., Keen, J. E., Siragusa, G. R., Barkocy-Gallagher, G. A., Koohmaraie, M., and Laegreid, W. W. (2000). Correlation of enterohemorrhagic *Escherichia coli* O157 prevalence in feces, hides and carcasses of beef cattle during processing. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 2999-3003.
Laegreid, W. W., Elder, R. O., and Keen, J. E. (1999). Prevalence of *Escherichia coli* O157:H7 in range beef calves at weaning. *Epidemiol. Infect.* **123**, 291-298.

2.4

PROCEEDINGS/ABSTRACTS:

Elder, R.O., Keen, J. E. Effects of pen cleaning and group vs. individual penning on fecal shedding of naturally-acquired enterohemorrhagic *E. coli* (EHEC) O157 in beef feedlot cattle. Proceedings, 80th Conference of Research Workers in Animal Diseases, Chicago, IL, November 7-9, 1999. Abstract Presentation No. 73.

Keen, J.E., Uhlich, G.A., Elder, R.O. Effects of hay- and grain-based diets on fecal shedding of naturally-acquired enterohemorrhagic *E. coli* (EHEC) O157 in beef feedlot cattle. Proceedings, 80th Conference of Research Workers in Animal Diseases, Chicago, IL, November 7-9, 1999. Abstract Presentation No. 86.

Uhlich, G. A. and Elder, R. O. Phase variation of curli expression in strains of *Escherichia coli* O157:H7. Proceedings, American Society of Microbiologists, Los Angeles, CA, May 20-24, 2000. Abstract p. 144.

CRIS Title: Control of Enterohemorrhagic *Escherichia coli* (EHEC) and Non-host Adapted *Salmonella* in Cattle
CRIS: 5438-42000-005
Scientist: Keen JE
Location: Animal Health Research Unit, MARC, Clay Center, NE
Contact: 402-762-4343 (P); 402-762-4375 (F); keen@email.marc.usda.gov

Summary Project Aims:

Foodborne disease is a major public health concern in the U.S. The gastrointestinal tracts of livestock serve as the source of some of the more important bacteria which cause foodborne diseases, such as *Escherichia coli* O157:H7 and *Salmonella typhimurium* DT104. Since livestock, especially cattle, appear to be major sources and vehicles of direct and indirect human infection with these pathogens, control and treatment of enterohemorrhagic *E. coli* (EHEC) and non-host adapted *Salmonella* (NHAS) infection in livestock should reduce the incidence of the foodborne human disease resulting from these bacteria.

Developing improved diagnostic methods for important foodborne bacterial species allows for very accurate studies of natural infections in commercial and experimental herds of cattle. Sources of infection, dynamics of infection and effects of external influences (such as management, environment, etc.) are identified as potential control points to reduce prevalence of these bacteria in livestock, thereby, improving the safety of the U.S. food supply.

Foodborne disease is one of the most important issues in public health and animal agriculture. Changes in the production, processing, distribution and consumption of food have contributed to the emergence of new pathogens and to the increasing importance of old ones. This has markedly influenced both the domestic and international markets for meat and related products in the U.S. Prevention and control strategies for foodborne pathogens must be designed and implemented at all levels of the production cycle, including the preharvest period, to reduce the risk to consumers and economic impacts on producers.

Determining how to reduce microbial pathogens in food products, throughout food operations from farm to fork, is the most urgent food safety problem today. A major goal of this program is to develop tests that are precise and rapid enough to detect contamination in all foods prior to their entering into commerce. Equally important is the development of effective, reliable and cost-effective methods to control or eliminate pathogens in/on food-producing animals throughout production and processing.

Using epidemiological surveillance, a better definition of the economic impact of livestock diseases can be gained to better understand the ecology of emerging diseases and natural transmission cycles. This is needed to develop control strategies to prevent disease. The emergence of a new disease or introduction of an exotic disease into the U.S. could rapidly escalate into an epidemic due to the lack of resistance in host animals, absence of vaccines or effective drugs and limited resources to effectively manage the spread of these pathogens.

2.6

Summary Accomplishments during Entire Project:
While this is a new CRIS proposal, it extends portions of CRIS 5438-42000-004. There have been four major accomplishments over the life of this project. First, we have shown that there is a clear and significant relationship between infection of live cattle with *E. coli* O157:H7 and contamination of beef carcasses. This relationship underpins the entire concept of preharvest food safety. Second, we have developed an unsurpassed panel of monoclonal antibodies for the detection and characterization of EHEC and *Salmonella*, including type, group and virotype-specific antibodies. Several of these antibodies have been formatted for diagnostic tests, including one test which is widely used in the U.S. meat industry. Third, we have described the epidemiology of infection of cattle with EHEC and *Salmonella typhimurium* DT104 throughout the production cycle. These studies have shown the differences in progression of infection between these types of bacteria in cattle and have partially quantified the transmission between infected and naive cattle, important parameters for designing preharvest control methods. Fourth, we have evaluated several potential intervention strategies to reduce fecal shedding of *E. coli* O157:H7. Results of these experiments will reduce utilization of plausible, but ineffective, control strategies for *E. coli* O157:H7 and have indicated strategies worthy of further development.

Summary 2000 Accomplishments:
This is a new CRIS project.

Projected Research Accomplishments during Next 3 Years:
During 2001-2002, we will complete at least four large anti-EHEC O157 field intervention trials utilizing naturally-infected feedlot cattle (i.e., 200 or more animals per trial, trial duration of one to three weeks and complete at least four experimental anti-*Salmonella* or anti-EHEC O157 intervention trials in a mouse model system). In 2003, we will complete at least two large field intervention trials utilizing naturally-infected finished feedlot cattle naturally infected with either non-O157 EHEC (O111 or O26) or NHAS (i.e., 200 or more animals per trial, trial duration of one to three weeks). We will also conduct validation trials on the most best potential anti-EHEC O157 control (prevalence reduction) techniques.

Technology Transfer:
Reduction of numbers of cattle infected with *E. coli* O157:H7 should significantly reduce the risk of contamination of the U.S. meat supply. Methods to reduce numbers of infected cattle should be evaluated in naturally infected animals under production conditions to minimize costs of instituting ineffective methods and to estimate unintended outcomes such as adverse health effects. These studies found that two plausible control strategies were either ineffective (high levels of pen sanitation) or associated with significant adverse health and economic effects. Furthermore, the inadvertent beneficial effect of transport was demonstrated. These results have been communicated to cattle producer groups directly (NCBA) and through the lay press (Beef, September 1999).

There has been great interest in this methodology among industry, research, academic, diagnostic and regulatory groups. Scientists from major meat packing companies, several universities, NVSL, NAHMS and other ARS researchers have been provided with the protocol and/or been trained in the use of this technique in our laboratories. Widespread use of culture techniques with greater sensitivity will significantly improve monitoring of infected livestock, evaluation of intervention strategies and detection of environmental contamination. As the recent outbreak of EHEC O111 in Texas demonstrated, serotypes other than O157:H7 represent threats to the U.S. food supply. Serotypes O26, O111 and O103, among others, are relatively more common elsewhere in the world and our studies have shown them to be present in U.S. cattle. Use of monoclonal antibodies to these O antigen types in rapid diagnostic and screening tests will provide and estimate of the potential risk to the U.S. meat supply. Constraints on the development of rational preharvest control strategies still remain--particularly, incomplete knowledge of the ecology and epidemiology of the relevant bacteria in a production setting.

PUBLICATIONS:

None

PROCEEDINGS/ABSTRACTS:

Keen, J.E., Elder, R.O. High but variable enterohemorrhagic *E. coli* (EHEC) O157 fecal shedding in pens of slaughter-ready Kansas and Nebraska beef feedlot cattle. Proceedings, 9th International Symposium on Veterinary Epidemiology and Economics, Breckenridge, CO, August 6-11, 2000. Abstrast Presentation No. 479.

Keen, J.E. Elder, R.O. High prevalence of enterohemorrhagic *E. coli* (EHEC) O157 on hide surfaces and in the oral cavity of finished beef feedlot cattle. Proceedings, 9th International Symposium on Veterinary Epidemiology and Economics, Breckenridge, CO, August 6-11, 2000. Abstract Presentation No. 480.

2.8

CRIS Title: Prevent Pathogen Contamination in Food Producing Animals, in particular, Cattle
CRIS: 6208-42000-001
Scientists: Morrow-Tesch, J, microbiologist (vacant)
Location: Livestock Issues Research Unit, Lubbock, TX
Contact: 806-742-4214 (P); 806-742-2335; julie.morrow@ttu.edu

Summary Project Aims:
Safety of animal food products is of paramount importance to the consumer. Meat, in particular, has received criticism because of bacterial contamination. Management methods based on animal behavior that reduce pathogen contact and shedding in production environments, during transportation and prior to slaughter need to be developed.

Summary Accomplishments during Entire Project:
Research was conducted to determine if methods of cooling feedlot cattle affect pathogen loads on their hides following transport to a processing facility. Studies on methods to reduce heat stress in feedlot cattle demonstrated that cooling cattle by misting them with water was associated with increased levels of the bacteria E. coli and E. coli 0157 on their hides following transport to a processing facility. Provision of shade reduced physiological and behavioral responses to heat stress while cooling cattle with water may increase the risk of pathogen contamination of food. The data is preliminary and studies to investigate the link between methods of reducing heat stress in cattle and pathogen contamination need to be continued. Reducing the number of organisms such as E. coli O157:H7 prior to processing at the slaughter facility could have a large impact on food safety.

Summary 2000 Accomplishments:
Research was conducted to determine if methods of cooling feedlot cattle affect pathogen loads on their hides following transport to a processing facility. Studies on methods to reduce heat stress in feedlot cattle demonstrated that cooling cattle by misting them with water was associated with increased levels of the bacteria E. coli and E. coli 0157 on their hides following transport to a processing facility. Provision of shade reduced physiological and behavioral responses to heat stress while cooling cattle with water may increase the risk of pathogen contamination of food. The data is preliminary and studies to investigate the link between methods of reducing heat stress in cattle and pathogen contamination need to be continued.

Projected Research Accomplishments during Next 3 Years:
In general the accomplishments will combine knowledge of animal behavior with physiological measurements to develop transportation and management practices for producers to use to help increase animal comfort and well-being and assure that animals will be presented for slaughter with few pathogens. Objective indicators of animal well-being will continue to be developed including acute phase proteins. We will utilize these measures to understand the associations between management, pathogen shedding, well-being, behavior, health, and productivity in cattle and pigs. This is dependent on either collaborating with ARS units with existing microbiology efforts (scientists, laboratory equipment) or the recruitment of a microbiologist into the Livestock Issues Research Unit. We will quantify stress responses of cattle and pigs to specific management practices

(including transportation) and to entire productions systems. Methods will include behavior, acute phase protein levels, and immune assays. We will continue to work with commercial feedlots to quantify production responses to heat stress and the effect of cooling strategy on pathogen loads in transported cattle.

Technology Transfer:
Constraints are primarily the cost of implementing new management systems for the producer. We still do not have the full compliment of ARS scientists needed to directly address pre-harvest food safety. Briefings with feedlot managers have been completed to inform them of our results and allow them to use management practices that reduce dust and heat stress.

PUBLICATIONS:

McGlone, J.J., von Borell, E., Curtis, S.E., Ford, J., den Hartog, L., Johnson, R., Meisinger, D., Morrow-Tesch, J.L., Mortensen, B., Reeves, D., Signoret, J-P, Sundberg, P., Thijssen, E., Tielen, M.J.M. Interpretive review of housing systems for gestating gilts and sows based on physiology, behavior and productivity. 2000. Report to the National Pork Producers

Council-Eicher, S.D., Morrow-Tesch, J.L., Albright, J.L., Dailey, J.W., Young, C.R., Stanker, L.H. Tail-docking influences on behavioral, immunological, and endocrine responses in dairy heifers. Journal of Dairy Science. 2000. 83:1456-1462.

PROCEEDINGS/ABSTRACTS:

Gentry, J.G., Blanton, J.R., McGlone, J.J., Morrow-Tesch, J.L., Miller, M.F. Pork quality and muscle characteristics of pigs finished indoors or outdoors during the winter months. J. Animal Sci. 2000. 78 (Suppl. 1):158.

Johnson, A.K., Morrow-Tesch, J.L., McGlone, J.J. Fender design and insulation effects of farrowing huts on productivity of outdoor sows and piglets. 2000. J. Animal Sci. 2000. 78 (Suppl. 1):237.

Morrow-Tesch, J.L., McGlone, J.J., Dailey, J.W., Anderson, D. Effect of transportation on young pigs. J. Animal Sci. 2000. 78 (1):35.

Scroggs, L.V., Kattesh, H.G., Morrow-Tesch, J.L., Stalder, K.J., Dailey, J.W., Schneider, J.F. 2000. Influence of split marketing on the physiology, behavior and performance of finishing swine. J. Animal Sci. 2000. v. 78 (1):34.

Swanson, J.C. Morrow-Tesch, J.L. Cattle transport: historical and future perspectives. 2000. Invited abstract at American Society Animal Science meetings, Baltimore, MD. Abstract #4

2.10

CRIS Title: Prevention of Losses from Colibacillosis and O157:H7 in Cattle and Swine
CRIS: 3625-32420-001
Scientists: Casey TA, Dean-Nystrom EA, Sharma VK
Location: Pre-Harvest Food Safety and Enteric Diseases Research Unit, National Animal Disease Center, Ames, IA
Contact: 515-663-7728 (P); 515-663-7458 (F); tcasey@nadc.ars.usda.gov

Summary Projects Aims:
Cattle are an important source of *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* which cause foodborne diseases in humans including bloody diarrhea, severe kidney disease, and sometimes death. Reducing the amount of *E. coli* O157:H7 in cattle should decrease the incidence of human disease caused by this bacteria. Other types of *E. coli* that are pathogenic for swine, cause severe diarrhea or edema disease in weaned pigs. These diseases are often fatal and have a significant economic impact on producers. There are no effective *E. coli* vaccines for diarrhea or edema disease in weaned pigs. Cattle inoculated with *E. coli* O157:H7 have been examined to find the location in the intestinal tract where these bacteria attach to intestinal cells and proliferate to form large colonies. Also, cattle have been inoculated with mutant O157:H7 *E. coli* to determine the role of specific bacterial genes in the disease and in bacterial attachment and colony formation. Based on this information, methods to reduce O157:H7 shedding in cattle have been tested. Rapid and sensitive diagnostic tests are being developed for the detection of *E. coli* O157:H7 in bovine tissues and fluids.

Summary Accomplishments During Entire Project:
E. coli O157:H7 was shown to cause severe, sometimes fatal disease, in experimentally inoculated newborn calves. Newborn calves developed diarrhea, had damaged intestines and shed large amounts of *E. coli* O157:H7 in feces. *E. coli* O157:H7 did not cause disease in 3- to 4-month-old weaned calves. However, weaned calves that were fasted 48 hours before inoculation with *E. coli* O157:H7 had intestinal damage and shed large amounts of *E. coli* O157:H7 in feces. In similar experiments, the *E. coli* O157:H7 surface molecule called intimin was required for colonization in both newborn and weaned calves. *E. coli* O157:H7 colonized and damaged the intestines of suckling and colostrum-deprived neonatal piglets but caused a more severe systemic disease in suckling pigs. These experiments led to the passive immunization experiments with intimin in suckling neonatal pigs described above. A diagnostic test, called multiplex polymerase chain reaction (PCR) was developed to identify genes that are important for *E. coli* O157:H7 colonization or for *E. coli* that cause disease in weaned pigs. A rapid and sensitive fluorescent method for detecting PCR products for the *E. coli* O157:H7 genes was developed which can be used to screen large numbers of samples simultaneously. A method and devices for detecting fecal contamination on carcasses in near-real time were developed, patented, and licensed for commercial development. These devices will be useful for reducing fecal contamination in food and decreasing the incidence of human foodborne diseases.

Summary 2000 Accomplishments:
Sensitive and rapid diagnostic tests for multiple foodborne pathogens are needed to improve food safety and reduce the human disease. ARS scientists at the NADC developed a new fluorescent polymerase chain reaction test which can simultaneously detect *Salmonella* and *E. coli* O157:H7. This test was very specific and could detect as few as 10 bacteria in contaminated meat or feces in about six hours. This diagnostic test could be used by the meat processing industry and FSIS to rapidly analyze large numbers of meat samples and to help prevent *Salmonella* and *E. coli* O157:H7 from entering the food supply. Previous experiments demonstrated that a surface molecule called intimin, was required for *E. coli* O157:H7 colonization and suggested that an immune response to intimin might prevent colonization and reduce fecal shedding. Scientists at NADC and Uniformed Services University of the Health Sciences (Bethesda, MD) collaborated to determine if neonatal piglets suckling sow vaccinated with intimin would be passively protected from experimental infection with *E. coli* O157:H7 and to determine if protection could be correlated with the amount of maternal antibody against intimin in colostrum. Piglets nursing sows with high amounts of anti-intimin antibody were protected from *E. coli* O157:H7 colonization while piglet nursing sows without anti-intimin antibody were colonized and had intestinal damage caused by *E. coli* O157:H7. These initial results are encouraging and suggest that intimin may be a useful target for an active immune response which will reduce or prevent *E. coli* O157:H7 colonization and fecal shedding.

Projected Research Accomplishments During Next 3 Years:
Passive immunization experiments with intimin will continue in suckling neonatal piglets and we will begin experiments to determine the effectiveness of intimin vaccination in bovine experimental models of *E. coli* O157:H7 infection. Fluorogenic PCR assays will be developed which target messenger RNA for specific *E. coli* O157:H7 genes and will make the test much more sensitive than current DNA-based assays. We anticipate that a reproducible model of *E. coli* O157:H7 infection in older calves will be developed which can be used to determine if intimin vaccination will prevent or reduce colonization and shedding of *E. coli* O157:H7 (experiments in FY 2003). We plan to construct specific *E. coli* O157:H7 mutant strains which do not express suspected virulence genes, such as stx and tir, and to determine if these genes affect colonization and shedding in experimentally inoculated animals. We anticipate that, in collaboration with our CRADA partners, we will have designed and will begin testing a large-scale fecal detection device that will be able to instantaneously detect contamination on an entire carcass and that this device or earlier hand-held devices will become commercially available. Conduct experiments to identify *E. coli* O157:H7 genes that are expressed only when the bacteria are growing within the intestinal tract of animals. These experiments may identify additional gene products that could be used in vaccines to prevent *E. coli* O157:H7 colonization and shedding.

Technology Transfer:
Information on the experimental *E. coli* O157:H7 infection models in calves and piglets developed in this project has been transferred to other scientists interested in *E. coli* O157:H7 pathogenesis, ecology, and vaccine development. The semi-automated fluorgenic PCR assay for rapid detection of *E. coli* O157:H7 and other Shiga toxin-producing *E. coli* has been transferred to state health laboratories, veterinary diagnostic laboratories, meat processing plants, and other scientists. The

fecal detection method and device has been patented by ARS and Iowa State University (U.S. Patent 5,914,247). This fecal detection technology has been licensed to an instrument design company and ARS and Iowa State University have entered into a CRADA with this company.

PUBLICATIONS:

Sharma, V. K., Carlson, S. A. Simultaneous detection of *Salmonella* strains and *Escherichia coli* O157:H7 with fluorogenic PCR and single-enrichment-broth culture. Appl. Environ. Microbiol. Accepted 9/11/00.

Cornick, N. A., Booher, S. L., Casey, T. A., Moon, H. W. Persistent colonized sheep by *Escherichia coli* O157:H7 and other *E. coli* pathotypes. Appl. Environ. Microbiol. Accepted 9/15/00.

Pelan-Mattocks, L. S., Kehrli Jr., M. E., Casey, T. A., Goff, J. P. Variable shedding of fecal coliform bacteria from periparturient dairy cows. Am. J. Vet. Res. Accepted 11/30/00.

Ashby, K. D., Casey, T. A., Rasmussen, M. A., Petrich, J. W. Steady-state and time-residual spectroscopy of F420 extracted from methanogen cells and its utility as a markers for fecal contamination. J. Agri. Food Chem. Submitted.

PROCEEDINGS/ABSTRACTS:

Sharma, V. K., Casey, T. A. Application of a multiplex PCR assay for simultaneous detection of Shiga toxigenic *Escherichia coli* and enterohemorrhagic *E. coli* of serotypes O157:H7, O26, and O111. 2000. 4th Int. Symp. and Workshop on "Shiga toxin (Verocytotoxin)-producing *Escherichia coli* infections.":Abstract p. 112.

Dean-Nystrom, E. A., Gansheroff, L., Twiddy, E., Moon, H., O'Brien, A. Passive protection of suckling piglets from *Escherichia coli* O157:H7 infection by vaccination of pregnant sows with $intimin_{O157}$. 2000. 4th Int. Symp. and Workshop on "Shiga toxin (Verocytotoxin)-producing *Escherichia coli* infections.":Abstract p. 175.

Sharma, V. K., Carlson, S. A. Simultaneous identification of *Salmonella* and enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 in feces and foods using an enrichment broth culture-fluorogenic multiplex PCR assay. 2000. 100th Gen. Meet. Am. Soc. Microbiol.:Abstract p. 514.

Sharma, V. K., Dean-Nystrom, E. A., Casey, T. A. Development of fluorogenic PCR assays for rapid detection of virulence genes of *Escherichia coli* O157:H7 and other shiga toxigenic *E. coli*. 1999. Virulence Mechanisms of Bacterial Pathogens Meet.:Abstract p. 17.

Cornick, N. A., Booher, S. L., Casey, T. A., Moon, H. W. Are ruminants a biological or accidental reservoir of VTEC O157? 1999. Pathogenicity and Virulence of Verotoxigenic *Escherichia coli*:Abstract p. 56.

Wunn, D., Andreasen, C., Carter, M., Ackermann, M., Nystrom, E. The use of digital imaging to assess bovine erythrocyte morphology. 1999. Am. Soc. Vet. Clin. Pathol.:Abstract p. 481.

2.14

CRIS Title: Microbial Factors-Pathogenesis of Sub-Acute Acidosis (SARA) in Cattle to Assure Food Safety
CRIS: 3625-32000-041
Scientists: Rasmussen M, Goff JP, Horst RL
Location: Periparture Diseases of Cattle Unit, National Animal Disease Center, Ames, IA
Contact: 515-663-7350 (P); 515-663-7669 (F); mrasmuss@nadc.ars.usda.gov

Summary Projects Aims:
The challenge of ensuring a safe food supply is the major problem addressed by this research program. There are two parts to this project: Identify and control food-borne pathogens in the gut of animals and on carcasses; and reduce exposure of humans and farm animals to toxins contained in food and feed supplies. The goals are to develop detection systems and control/intervention strategies that will decrease the risk of human food-borne illness and enhance animal productivity and health.

Summary Accomplishments During Entire Project:
Invention, development and commercialization of a fecal detection system based upon fluorescent spectroscopy. This technology will be used in slaughter plants for the real-time detection of carcass contamination. Systems will be designed for scanning whole carcasses and for spot checking smaller areas on cuts of meat. The technology will help the packing industry produce cleaner products and assist with HACCP and zero tolerance requirements.

Summary 2000 Accomplishments:
We developed a new way to help detect fecal contamination, thereby helping keep meat carcasses clean during processing. Technology will change the way packing plants detect contamination and keep carcasses clean during processing.

Projected Research Accomplishments During Next 3 Years:
We will, through our industrial partner (eMerge Interactive, Sebastion, FL), design and construct commercially useful instruments. We anticipate that we will complete development of the fecal detection system for the beef industry within the next year. We will then continue development of this technology so it is applicable to the pork and poultry industries. We are also initiating a new project to devise methods to control lactic acidosis in cattle.

Technology Transfer:
We transferred the fecal detector technology and established a license and CRADA between ARS, Iowa State University, and a commercial partner, eMerge Interactive (Sebastion, FL). The fecal detection technology is now under commercial development by engineers at eMerge. Commercial instruments for industry are anticipated within 18 months, according to eMerge estimates. Current technology is designed for beef processing. Further development as necessary to adopt technology to pork and poultry processing.

PUBLICATIONS:

Anderson, R C., Rasmussen, M. A., Jensen, N. S., and Allison, M. J. Denitrobacterium detoxificans gen. nov., sp. nov., a ruminal bacterium that respires on nitrocompound. International Journal of Systematic Evolutionary Microbiology. 2000. March 50 (2) p. 633-638.

PROCEEDINGS/ABSTRACTS:

Ashby, K.D., Casey, T.A., Petrich, J.W., Rasmussen, M.A. Detection of fecal and ingesta contamination on meat surfaces utilizing intrinsic fluorescent markers. 1999. Abstr. North Central Br. ASM mtg. p. 21.

Koenigsfeld, M.J., Rasmussen, M.A., Casey, T.A. Antimicrobial effect of acidified nitrite on Escherichia coli O157:H7. 1999. Abstr. North Central Br. ASM mtg. p. 22.

CRIS Title: Microbial Competitive Exclusion to Reduce Epizootic Pathogenic Bacteria in Swine and Cattle
CRIS: 6202-42000-010
Scientists: Anderson RC, Beier RC, Callaway TR, Harvey RB, Hume ME, Nisbet DJ
Location: Food and Feed Safety Research Unit; SPARC, College Station, TX
Contact: 979-260-9317 (P); 979-260-9332 (F); anderson@ffsru.tamu.edu

Summary Project Aims:
Swine and cattle carry harmful microorganisms in their digestive tracts. Some of these microbes cause production losses such as reduced weight gains and death (particularly of young animals) and can contaminate meat products intended for human consumption. Microbial food poisoning resulting from consumption of contaminated products can cause serious human illness, costing the United States more than $4 billion annually in medical costs and lost wages. These illnesses can be severe enough to cause death, especially among the young, elderly or those with weakened immune function. Our research focuses on reducing the incidence, survivability, and virulence of important microbial foodborne pathogens in the gut of swine and cattle during all stages of live animal production. This work is of importance to all involved in the production, processing, distribution, preparation, and consumption of food products derived from swine and cattle. Success in the work will provide new technologies and intervention strategies that will allow production of safer meat products and significantly lower incidences of food poisoning for consumers of American meat products.

Summary Project Accomplishments During Entire Project:
This is a relatively new project whose major focus is on development of competitive exclusion (CE) and other approaches for effective reduction of gut colonization in swine and cattle by pathogenic microorganisms that cause food poisoning in humans. Such technology, once adapted to commercial production, will enhance the microbiological safety of pork and beef reaching the consumer as well as protection of young pigs and calves from the lethal effects of pathogenic *E. coli* and *Salmonella*. Additionally, significant accomplishments have been made in the development of models of swine and ruminant gut microbiology. These models will greatly facilitate our research and that of others in better understanding the microbial ecology within the gut and will facilitate the development of technologies that maintain healthy normal flora. We have already defined a number of physiological characteristics limiting the competitiveness, survivability, and virulence of *Salmonella*, *Campylobacter*, and pathogenic *E. coli.*

Summary 2000 Accomplishments:
The swine and cattle industries need new technologies to eliminate pathogenic microorganisms that live in the animal gut and that can cause morbidity or death, particularly in young animals. In cooperation with industry partners, in-house research efforts and field tests at commercial Texas swine facilities have resulted in the development and patenting of a defined porcine-derived competitive exclusion (CE) culture that is effective in enhancing colonization resistance and

survivability of piglets exposed to *Salmonella* and pathogenic *E. coli*. This CE culture has been recognized as a new animal drug, is currently being tested in commercial swine herds, and negotiations for licensing are underway with our CRADA partner.

One focus of our in-house research is to identify and(or) develop additives that can be fed to cattle or pigs to selectively kill pathogenic gut microorganisms but not harm beneficial gut microorganisms. We have found that chlorate added to drinking water results in selective killing of *Salmonella* and pathogenic *E. coli* but not beneficial gut bacteria. Chlorate supplementation should be a commercially viable strategy for livestock producers to reduce concentrations of gut pathogens thus enhancing the microbiological safety of meat products derived from swine and cattle.

In order to more clearly understand the various ecological factors affecting the ability of foodborne pathogens survive and persist within the gut environment, we are studying the microbial ecology within the gastrointestinal tracts of cattle and pigs. Using state-of-the-art continuous-flow culture methodology, we have developed and validated models of ruminant and pig gut microbiology. Results indicate that the subtherapeutic amounts of the commonly used antibiotic chlortetracycline provided little if any extra benefit to a healthy gut floras ability to resist colonization by *Salmonella.* These results suggest that strategies that facilitate the establishment and maintenance of a healthy normal flora within the gut of young animals may prevent and reduce enteropathogen colonization thus obviating the need for subtherapeutic antibiotic use.

A need exists to identify stages in animal production where implementation of preharvest pathogen reduction strategies can be most effective. In collaboration with Texas A&M University and with CRADA partners, we studied the natural colonization patterns of swine by *Campylobacter* and *Salmonella.* We demonstrated that early colonization of piglets by these foodborne pathogens plays a major role in the maintenance and dissemination of the pathogens within the production environment. These results suggest that successful comprehensive control of foodborne pathogens will include strategies targeted to prevent infections in young animals.

Campylobacter is the most often cause of foodborne illness, but much remains to be learned regarding how to prevent the colonization of food producing animals by this pathogen. In order to learn more about how swine are colonized by *Campylobacter*, a survey of *Campylobacter* in a swine population was conducted. Using polymerase chain reaction (PCR) amplication and pulsed field gel electrophoresis for genotypic strain identification, we have determined that multiple *Campylobacter* genotypic strains can exist in an individual animal at the same time and that weaned pigs can be colonized by additional genotypic strains other than those isolated from their maternal sows. Thus, while maternal transmission plays a critical initial role in colonization, horizontal transfer between pen mates readily occurs postweaning.

Genotypic strain identification and PCR were extended to potentially pathogenic *Arcobacter* species. A survey of porcine *Arcobacter* isolates collected from pigs from a farrow-to-finish operation indicated that the incidence of arcobacteriosis of the digestive tract increased as pigs progressed from the farrowing barn to the finishing barn, where 100% of the finishing pens were *Arcobacter*-positive.

Studies with *Arcobacter* were extended to the on-going development of a PCR-derived genetic probe for the rapid detection and enumeration of *Arcobacter* in porcine fecal and cecal materials. Information obtained from these studies will be useful for developing technologies to prevent *Arcobacter* colonization in swine.

Projected Research Accomplishments During Next 3 Years:
Negotiations and exclusive licensing of the defined porcine competitive exclusion culture product will be completed. Field trials will be initiated and data will be submitted to the FDA for evaluation. A CRADA partner will be identified for our chlorate technology development, and producer-supported chlorate research will be completed. Substrate-adapted CE cultures targeted at displacing pre-existing pathogen infections in swine will be developed. Field trials with chlorate will begin and will include effects of chlorate on carcass characteristics. Work on substrate-adapted CE cultures will be done in live animals. FDA field trials with the porcine CE culture will be completed. FDA field trials to support the chlorate technology will be initiated. Commercial field trials with the substrate-adapted CE culture technology will be initiated in swine.

Technology Transfer:
The swine CE culture technology is well developed and patent protection of this culture has been granted (patent number 5,951,977). No constraints to the effective application of CE technology are known and it should be a commercial product within two years. The chlorate technology is being tested in cooperation with a producer organization. There does not appear to be any significant constraint to ultimate commercial application of the chlorate technology and it is anticipated that it will be in the marketplace within three years.

PUBLICATIONS:

Anderson, R.C., Buckley, S.A., Kubena, L.F., Stanker, L.H., Harvey, R.B., Nisbet, D.J. Bactericidal effect of sodium chlorate on *Escherichia coli* 0157:H7 and *Salmonella* Typhimurium DT104 in rumen contents in vitro. Journal of Food Protection. 2000. v. 63. p. 1038-1042.

Anderson, R.C., Genovese, K.J., Harvey, R.B., Stanker, L.H., DeLoach, J.R., Nisbet, D.J. Assessment of the long term shedding pattern of *Salmonella* serovar *choleraesuis* following experimental infection of neonatal piglets. Journal of Veterinary Diagnostic Investigation. 2000. v. 12. p. 257-260.

Genovese, K.J., Anderson, R.C., Nisbet, D.J., Harvey, R.B., Lowry, V.K., Buckley, S., Stanker, L.H., Kogut, M.H. Prophylactic administration of immune lymphokine derived organ invasion and cecal colonization in weaned pigs. Paul, P.S., Francis, D.H., editors. Kluwer Academic/Plenum Publishers, New York, NY. Mechanisms in the Pathogenesis of Enteric Diseases 2. 1999. p. 299-307.

Harvey, R.B., Anderson, R.C., Young, C.R., Hume, M.E., Genovese, K.J., Ziprin, R.L., Farrington, L.A., Stanker, L.H., Nisbet, D.J. Prevalence of *Campylobacter, Salmonella*, and *Arcobacter* species at slaughter in marked age pigs. Paul, P., Francis, D.,(ed). Kluwer Academic/Plenum Publishers, New York, NY. Mechanisms in the Pathogenesis of Enteric Diseases 2. 1999. p. 237-239.

Harvey, R.B., Young, C.R., Ziprin, R.L., Hume, M.E., Genovese, K.J., Anderson, R.C., Droleskey, R.E., Stanker, L.H., Nisbet, D.J. Prevalence of *Campylobacter* spp. isolated from the intestinal tract of pigs raised in an integrated swine production system. Journal of the American Veterinary Medical Association. 2000. v. 215. p. 1601-1604.

Young, C.R., Harvey, R.B., Anderson, R.C., Nisbet, D.J., Stanker, L.H. Enteric colonization following natural exposure to *Campylobacter* in pigs. Research in Veterinary Science. 2000. v. 68. p. 75-78.

Nisbet, D.J., Corrier, D.E., Stanker, L.H. Competitive exclusion culture for swine. 1999. U.S. Patent 5,951,977.

PROCEEDINGS/ABSTRACTS:

Anderson, R.C. Potential uses for competitive exclusion products. In: Subtherapeutics, probiotics, alternatives. 26th Annual Allen D. Leman Swine Conference, College of Veterinary Medicine, University of Minnesota. 1999. p. 53-65.

Harvey, R.B., Genovese, K J., Anderson, R.C., Nisbet, D.J. 2000. Competitive exclusion and its potential for improved pork safety. In: Proceedings, Pork Quality and Safety Summit, National Pork Producers Council. July, 2000. p.313-317.

Nisbet,D.J., Anderson, R.C., Genovese, K.J. Competitive exclusion in swine. *In* Proc. 15th Annual Carolina Swine Nutrition Conf., Raleigh, NC, Nov. 8-9, 1999. p. 25-37.

CRIS Title: Salmonella-Host Interactions
CRIS: 3625-42000-006
Scientists: Stabel TJ
Location: Pre-Harvest Food Safety and Enteric Diseases Research Unit, National Animal Disease Center, Ames, IA
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Summary Project Aims:
Salmonella typhimurium, the most ubiquitous of all Salmonellae, is the leading cause of food poisoning in humans. Swine, along with poultry, cattle, and seafood, are important reservoirs of *Salmonella*. Current efforts to identify and eradicate animals that are persistently infected with *Salmonella* have been impeded by a lack of information regarding the epidemiology, immunology, pathogenesis, and host specificity of salmonellosis. A related problem has been the increased risk of antibiotic resistant *Salmonella* to the human population. The mission of our research program is to develop new technologies, through basic and applied science, for management and control of *Salmonella* on the farm and in the slaughter plant. Our program has four major objectives: (1) determine the epidemiologic factors that may be subjected to intervention measures for control of *Salmonella* in swine, (2) define the immune response associated with acute and chronic *Salmonella* infection of swine, (3) monitor *Salmonella* isolates from cattle and swine for *Salmonella typhimurium* DT104, and (4) characterize the epidemiology, transmission, and nature of antibiotic resistance of the organism.

Summary Accomplishments During Entire Project:
In conjunction with a commercial pig breeder, an assay to measure the ability of pig macrophages to kill *Salmonella* was developed and tested. Using this assay, the ability of macrophages to kill *Salmonella* was shown to vary significantly between pigs. If there is a correlation between macrophage results and degree of sickness following *Salmonella* infection, development of a *Salmonella*-resistant line of pigs may be possible. Using an experimental stress model, stress factors that affect the immune system and salmonellosis in swine have been studied. Data suggests that pigs with salmonellosis can be divided into two categories, one group that is unaffected by stress and another group in which stress causes more severe disease and bacterial shedding. The group which is affected by stress could be an important source in foodborne outbreaks of *Salmonella*. A DNA-specific diagnostic test for S. typhimurium DT104 was developed and is currently being tested as a rapid tool for detection of contaminants in food and the environment. A quick and sensitive method for detection of S. typhimurium DT104, an important food safety bacteria, would be useful to slaughter plants and federal agencies. The use of ineffective penicillin-like antibiotics was shown to exacerbate the antibiotic resistance profile of multi-resistant S. typhimurium DT104. These findings will provide important insight for practitioners and regulatory agencies in the proper use of antibiotics on the farm. Multi-resistant S. typhimurium DT104 were found to be no more invasive than its non-resistant counterpart, providing further evidence that the multi-resistant bacteria is not a hyperinvasive superpathogen. Public information regarding the nature of multi-resistant *S. typhimurium* DT104 has been lacking and this new scientific data will provide sound background for future policy decisions. A simple rapid two-color antibody diagnostic test to identify immune

cell abnormalities in pig whole blood was developed using flow cytometry. Detection of common cell surface markers allows reproducible and accurate sampling of a large number of pigs in < 2 hours. This rapid test will benefit veterinary research and diagnostic laboratories. Scientists at the NADC combined two DNA-based tests to produce a single easy-to-use assay for the detection of *Salmonella typhimurium* DT104 and E. coli O157:H7. This rapid, sensitive and specific PCR-based test will allow for better detection of two important foodborne pathogens. Studies to determine the cellular interaction and adherence of *Salmonella* in the swine gut-loop model showed that *Salmonella* not only adhere to M-cells but also to sites of enterocytes extrusion. This potential virulence mechanism may have important implications for prophylactic treatment of salmonellosis in swine.

Summary 2000 Accomplishments:
The pig industry and federal regulators have expressed interest in a test that would detect major foodborne pathogens. We combined two DNA-based tests to produce a single easy-to-use assay for the detection of *Salmonella typhimurium* DT104 and *E. coli* O157:H7. This rapid, sensitive, and specific PCR-based test will allow for better detection of two important foodborne pathogens. There has been considerable debate as to the effect of antibiotic treatment on bacterial populations. We studied the invasive status of *Salmonella* after antibiotic treatment. It was determined that antibiotic treatment had no effect on the invasion potential of *Salmonella*. Information on the effects of antibiotic treatment on foodborne pathogens, such as *Salmonella*, is critical to development of effective public policy. Further tested the hypothesis that *Salmonella* initiate disease in swine by attacking and damaging specialized cells in the intestine called M-cells. Using scanning electron microscopy, we are currently studying the disease process associated with *S. typhimurium* DT104 and other serotypes, such as *S. choleraesuis*. Examination of tissues from small intestine gut-loops indicates that *S. typhimurium* DT104 preferentially adheres to M-cells and to sites of enterocyte cell death. Identification of novel mechanisms in bacteria-host specificity and pathogenesis will allow the development of more effective vaccines. Research and diagnostic labs have a common need for more rapid methods to analyze large numbers of blood samples. We have further developed a simple rapid two-color antibody phenotyping assay for porcine whole blood to identify common cell surface markers. Assay validation studies were conducted using a large number of pigs and assay results were consistent with reported values obtained using other more complicated techniques. A rapid diagnostic test for blood cell abnormalities is a potential outcome of this new technique benefiting the field of veterinary medicine.

Projected Research Accomplishments During Next 3 Years:
Using flow cytometry, identify changes in immune cell populations after infection with *Salmonella* spp. Develop an ELISA to measure blood sTNFR-1 during and after *Salmonella* infection. Repeat studies to evaluate the effects of experimental stress on the *Salmonella* carrier-state in swine. Identify polymorphisms in candidate genes for disease resistance and correlate with phenotypic data (macrophage oxidative burst potential, bacterial load in tissues, clinical signs, etc.). Evaluate the effect(s) of stress status on *Salmonella* infectivity and measure neuroendocrine response in stressed and/or infected pigs. Conduct porcine gene expression studies to identify differences in host-specificity between *S. typhimurium* and *S. choleraesuis* early and late after infection. Design and

conduct transportation stress studies with the USDA, ARS, Livestock Behavior Research Unit in West Lafayette, IN. Determine the effect of stress on the quantity and distribution of porcine intestinal immune cells before and after inoculation with *Salmonella.*

Technology Transfer:
A *S. typhimurium* DT104-specific multiplex/fluorogenic PCR was transferred to an ARS scientist for the purpose of using this detection method on foods and clinical fecal samples. As an appointed member of the National Pork Producers Council (NPPC) Salmonella Measurement Group, we participated in a round-table discussion of standard measurements that might be implemented in the in pork industry to assure a safe product. This meeting (May 31, 2000) brought together swine researchers from across the United States. Our research was presented to FSIS at the annual ARS/FSIS Food Safety Meeting held in Beltsville, MD, December 1999.

PUBLICATIONS:

Stabel, T. J., Bolin, S. R., Pesch, B. A., Rahner, T. E. A simple and rapid flow cytometric method for detection of porcine cell surface markers. 2000. J. Immunol. Methods v. 245 (1-2). p. 147-152.

Carlson, S. A., Browning, M., Ferris, K. E., Jones, B. D. Identification of diminished tissue culture invasiveness among multiple antibiotic resistant *Salmonella typhimurium* DT104. 2000. Microb. Pathog. v. 28 (1). p. 37-44.

Carlson, S. A., Willson, R. M., Crane, A., Ferris, K. E. Evaluation of invasion-conferring genotypes and antibiotic-induced hyperinvasive phenotypes in multiple antibiotic resistant *Salmonella typhimurium* DT104. 2000. Microb. Pathog. v. 28 (6). p. 373-378.

PROCEEDINGS/ABSTRACTS:

Stabel, T. J., Bolin, S. R., Pesch, B. A., Rahner, T. E. A simple and rapid flow cytometric method for detection of porcine cell surface markers. 2000. Am. Assoc. Immunol.:Abstract p. A1236.

Carlson, S. A., Wilson, R. L., Omary, M. B. and Jones, B. D. Identification of cytokeratins as potential mediators of *Salmonella* invasion. 1999. Virulence Mechanisms of Bacterial Pathogens Meet.:Abstract p. 16.

Stabel, T. J., Mwangi, S. M., Maroushek-Boury, N., Taylor, M. J. Development and characterization of monoclonal antibodies to porcine recombinant soluble tumor necrosis factor receptor 1. 1999. 80th Conf. Res. Workers Anim. Dis.:Abstract p. 18.

3.8

CRIS Title: Ecology and Epidemiology of *Salmonella* and other Foodborne Pathogens in Livestock, Primarily Swine
CRIS: 3625-42000-005
Scientists: Wesley IV, Hurd HS, Ziemer CJ
Location: Pre-Harvest Food Safety and Enteric Diseases Research Unit, National Animal Disease Center, Ames, IA
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Summary Projects Aims:
Salmonella, *Campylobacter jejuni*, and *Listeria monocytogenes* are major bacterial foodborne pathogens causing ~4 million human cases annually and accounting for an estimated $2.75 billion in human and medical productivity losses. The USDA nationwide microbial baseline survey of livestock and poultry showed high levels of microbial contamination on hog carcasses. Our research will focus on sampling isolation, identification, and quantification of foodborne pathogens in livestock and their environment. Rapid and sensitive second generation PCR-based techniques will be designed for detecting and enumerating *Salmonella*, *Campylobacter*, *Listeria*, and emerging bacterial agents (e.g., *Yersinia*).

The dynamics of environmental contamination on microbial carrier status and ultimately hog carcass contamination will be evaluated. Specific management practices to reduce the prevalence of zoonotic pathogens in livestock have not yet been identified. We are studying the epidemiology of these organisms on the farm, during transport, and at the abattoir, up to stunning. This study includes several aspects such as animal-to-animal transmission, environment-to-animal transmission, environmental sources, and reservoir hosts. This ecological perspective is expected to identify opportunities for specific control interventions.

Summary 2000 Accomplishments:
The CRIS, which was initiated June 2000, is a compilation of CRIS 3625-32420-004, 3625-42000-001, and 3625-42000-002.. A prospectus delineating the major objectives and the drafting of the 5-year CRIS project statement were accomplished since the inception of this project in June 2000.

Summary 2000 Accomplishments:
Holding at the abattoir is associated with increased *Salmonella enterica* isolation from market swine. Transportation and long-term (> 12 h) holding of swine prior to slaughter have been associated with an increase in *Salmonella* prevalence at the packing plant. This increase in prevalence has been attributed to the effects of stress, increased mixing of animals, and reoccurrence of shedding. Little mention has been made regarding the environment as a source of *Salmonella* infection. Currently, most U.S. abattoirs try to avoid holding pig more than 6-8 hours. However, a minimum of 2 hours is encouraged to improve meat quality. We have completed four studies in collaboration with Iowa State University College of Veterinary Medicine. These studies suggest that short-term (2-3 hours) holding at the abattoir may be a significant source of *Salmonella* infection. In the first study using pigs from one commercial producer, we noted a large increase (10-fold) in the proportion of pigs positive at slaughter (ileocecal lymph node, cecal content, distal colon content), compared to when

they were tested on the farm. This increase occurred in groups that were housed for an additional 18 hours at an off-site, cleaned and disinfected holding facility as well as those shipped directly to slaughter (85 miles). The lack of holding effect and the variety of *Salmonella* serotypes found at slaughter (n=17), compared to the farm (n=1), suggested that an external source of infection was more important than stress for increasing *Salmonella* isolation rates. To address differences in samples collected on-farm (feces) and after slaughter (ileocecal lymph node, cecal content, distal colon content), we conducted the second study using six herds enrolled in the Accelerated Pseudorabies Eradication Program. Market pigs (penmates) were randomly assigned to be necropsied on the farm of origin or at the abattoir, after transport in disinfected trailers and 2-3 hours holding. The same samples (feces, ileocecal lymph node, cecal content, superficial inguinal lymph node) were collected at both locations. For five of the six farms in this study, the isolation rates were significantly ($p < 0.05$) higher for penmates necropsied after transport and holding. Overall the average *Salmonella* isolation rate was five times higher ($p < 0.001$). The number of different serotypes isolated was much higher for those samples collected at the abattoir (n=17) compared to the farm (n=8).

The objective of the third study was to evaluate the possibility of swine becoming experimentally infected with *Salmonella,* in a 2-3 hour interval, from an environment contaminated with *Salmonella.* The environment was designed to simulate the antemortem holding pen. Forty crossbred market weight swine (~92 kg) were exposed to feces containing a nalidixic acid-resistant strain of *Salmonella enterica* Typhimurium. The contaminated feces were deposited on the floor by swine that had been intranasally inoculated 4 days previously. Exposed pigs were autopsied at 2, 3, and 6 hours after exposure to the contaminated floor. Within 2 to 3 hours, most (80%) of exposed animals were positive for the nalidixic acid-resistant strain. After 6 hours, all of the animals had at least one tissue sample test positive. This study shows that market swine can become infected with *Salmonella* in the short waiting time before slaughter.

In our fourth study, we swabbed floors of holding pens in two large commercial abattoirs. Over 80% of the pens yielded *Salmonella*. Positive samples included water samples from the pen water troughs. In combination, these four studies suggest that intervention at the holding pen may reduce the number of swine carrying *Salmonella* onto the kill floor. This reduction may have a significant impact on the safety of pork products.

Projected Research Accomplishments During Next 3 Years:
Develop rapid and sensitive molecular-based techniques to detect, quantitate, and characterize bacterial foodborne pathogens in livestock and their environment. Pathogens include *Salmonella*, *Campylobacter*, *Listeria*, and emerging bacterial agents (e.g., *Yersinia*). Delineate the dynamics of foodborne pathogens in livestock and their environment and develop intervention strategies to reduce transmission. Determine the pre-harvest food safety risk factors in livestock by correlating on-farm management practices with the presence of these foodborne agents. Management practices include feeding, transport, holding, biosecurity, housing, etc.

Technology Transfer:
Information on the effects of holding at the abattoir on *Salmonella* colonization is being transferred to abattoir management, veterinary practitioners, producers, and other researchers. We are currently in discussions with two major integrated producer packers to implement research studies using their animals and facilities. We have been asked to provide input into the design of a new abattoir and holding area for one of these companies.

PUBLICATIONS:

Wesley, I. V., Wells, S. J., Harmon, K. M., Green, A., Schroeder-Tucker, L., Glover, M., Siddique, I. Fecal shedding of *Campylobacter* and *Arcobacter* spp. in dairy cattle. 2000. Appl. Environ. Microbiol. v. 66 (5). p. 1994-2000.

Clark, E. E., Wesley, I., Fiedler, F., Promadej, N., Kathariou, S. Absence of serotype-specific surface antigen and altered teichoic acid glycosylation among epidemic-associated strains of *Listeria monocytogenes*. 2000. J. Clin. Microbiol. v. 38 (10). p. 3856-3859.

Jourdan, A. D., Johnson, S. C., Wesley, I. V. Development of a fluorogenic 5' nuclease PCR assay for the detection of the *ail* gene of pathogenic *Yersinia enterocolitica*. 2000. Appl. Environ. Microbiol. v. 66 (9). p. 3750-3755.

Wesley, I. V., Baetz, A. L. Natural and experimental infections of Arcobacter in birds. 1999. Poultry Sci. v. 78 (4). p. 536-545.

Oliveira de, S. J., Wesley, I. V., Baetz, A. L., Harmon, K. M., Kader, I., Uzeda, M. Arcobacter cryaerophilus and Arcobacter butzleri isolated from preputial fluid of boars and fattening pigs in Brazil. 1999. J. Vet. Diagn. Invest. v. 11 (5). p. 462-464.

Wesley, I. V. Arcobacter. Robinson, R., Batt, C. and Patel, P., editors. Academic Press Ltd., London. Encyclopedia of Food Microbiology. 1999. p. 50-54.

Hurd, H. S., McCluskey, B. J., Mumford, E. L. Management factors affecting the risk for vesicular stomatitis in livestock operations in the western United States. 1999. J. Am. Vet. Med. Assoc. v. 215 (9). p. 1263-1268.

McCluskey, B. J., Hurd, H. S., Mumford, E. L. Review of the 1997 outbreak of vesicular stomatitis in the western United States. 1999. J. Am. Vet. Med. Assoc. v. 215 (9). p. 1259-1262.

PROCEEDINGS/ABSTRACTS:

Wesley, I. V., Harmon, K. M., Green, A., Bush, E., Wells, S. Distribution of Campylobacter and Arcobacter in livestock. 2000. 1999 Swine Res. Rep. p. 211-216.

Wesley, I. V., Baetz, A. L., Laufer, J. A. The cannulated pig: A model for monitoring the dynamics of foodborne pathogens in vivo. 2000. 1999 Swine Res. Rep. p. 217-222.

Boyapalle, S. K., Wesley, I. V., Reddy, P. G. Comparison of a multiplex and 5' nuclease PCR assays for the rapid detection of pathogenic Yersinia enterocolitica in swine and pork products. 2000. 1999 Swine Res. Rep. p. 202-206.

Kanuganti, S., Wesley, I. V., Reddy, P. G. Rapid detection of Listeria monocytogenes in food and food animals. 2000. 1999 Swine Res. Rep. p. 207-210.

Hurd, H. S., McKean, J. D., Wesley, I. V., Karriker, L. A. The effect of lairage and transport on intestinal and meat prevalence of Salmonella in market weight pigs. 2000. Am. Assoc. Swine Practitioners: Abstract p. 429-434.

Wesley, I.V. Campylobacter. Soc. Ind. Microbiol: Food-borne Pathogens 2000 - Abstract p. 20.

Boyapalle, S., Kanuganti, S., Wesley, I. V., Hurd, H. S., Reddy, P. G. Comparison of a multiplex and 5' nuclease PCR assays for the rapid detection of pathogenic Yersinia enterocolitica in swine and pork products. Soc. Ind. Microbiol. Food-borne Pathogens 2000 - Abstract p. 26.

Hurd, H. S., McKean, J. D., Rostagno, M., Griffith, R., Wesley, I. *Salmonella* survey tools in swine: Farm and abattoir - preliminary report. 2000. Ann. Meet. Food Safety Consortium. p. 53-59.

Wesley, I. V., Wells, S., Harmon, K., Green, A. Risk factors for Campylobacter and Arcobacter in dairy cattle. 1999. 80th Conf. Res. Workers Anim. Dis.:Abstract p. 76.

Kanuganti, S., Wesley, I. V., Hurd, H. S., Karriker, L., McKean, J. D., Boyapalle, S., Reddy, P. G. Rapid detection of Listeria monocytogenes in food and food animals. 1999. 80th Conf. Res. Workers Anim. Dis.:Abstract p. 77.

Boyapalle, S., Wesley, I.V., Hurd, H S., Karriker, L., Kanuganti, S., McKean, J.D., Okwumabua, O., Reddy, P. G. Comparison of a conventional multiplex and 5' nuclease PCR assays for the rapid detection of pathogenic Yersinia enterocolitica in swine and pork products. 1999. 80th Conf. Res. Workers Anim. Dis.:Abstract p. 78.

Hurd, H. S., McKean, J. D., Wesley, I. V., Karriker, L. A. The effect of lairage and transport on intestinal and meat prevalence of Salmonella in market weight pigs. 1999. 80th Conf. Res. Workers Anim. Dis.:Abstract p. 96.

CRIS Title: Immunomodulation of the Avian Innate Immune Response to Prevent Bacterial Infections in Poultry
CRIS: 6202-42000-008
Scientists: Kogut MH, Crippen TL, He H
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Summary Project Aims:
Salmonella and Campylobacter are the leading causes of human food-borne illness caused by bacteria. There are an estimated 2-4 million cases of bacterially-derived human gastroenteritis treated each year, costing the United States over $4 billion annually in lost wages and medical costs. Poultry meat products are a major source of human food borne illness caused by both Salmonella and Campylobacter. Our research attempts to develop non-traditional, non-antibiotic, immunologically based strategies to prevent bacterial diseases in poultry. The work focuses on up regulating the birds' own host defenses during the first week after hatch which is the period of highest susceptibility.

Summary Accomplishments During Entire Project:
We have shown that naturally occurring poultry biochemicals known as cytokines protect young poultry against salmonellosis, and we have developed a procedure to modify poultry T lymphocyte cells such that they effectively protect young chicks against Salmonella enteriditis (SE). This protection can be attained by several different methods of bird treatment. We have made major progress in determining the mechanisms by which poultry can attain resistance to colonization by Salmonella and other pathogenic microorganisms.

Summary 2000 Accomplishments:
The poultry industry needs new intervention strategies to control pathogenic microorganisms that live in the gastrointestinal tract of birds. We discovered a protein that has antibacterial activity against Salmonella. The gene that produces this protein has been cloned. We believe we can use this discovery to develop natural products that will be able to effectively stimulate the innate defense mechanisms of young poultry, preventing colonization by pathogenic microorganisms such as Salmonella, and ultimately resulting in safer poultry food products reaching the consumer.

Projected Research Accomplishments During Next 3 Years:
Further define immune responsiveness of poultry at time of hatch and will exploit appropriate biochemical components as biomarkers of immune competency. Develop molecular probes to facilitate work on how Salmonella, Escherichia coli, and Campylobacter interact with the immune system of various poultry species. Develop gene transfer technology for use in stimulating the immune responses of birds so as to protect them from colonization by microbes such as Salmonella, E. coli, and Campylobacter.

4.2

Technology Transfer:
One of the products we have identified that protects poultry against pathogenic microbes (referred to as SILK) has been provided to other scientists both in the U.S. and abroad for use in research on the prevention and control of various viral, bacterial, and protozoal diseases of poultry. The main constraint for the ultimate commercial application of SILK technology is the purification and identification of the active molecules in SILK. Once we fully identify the components of SILK, the pharmaceutical industry will without question license this material. In addition, we have developed a CRADA with a poultry breeder to evaluate the effect of this technology in commercial birds. We anticipate, within 3-4 years, success in application of the SILK technology in commercial poultry production.

PUBLICATIONS:

Kogut, M.H., Genovese, K.J., Stanker, L.H. Effect of induced molting on heterophil function in white leghorn hens. Avian Diseases. 1999. v. 43. p. 538-548.

Genovese, K.J., Anderson, R.C., Nisbet, D.J., Harvey, R.B., Lowry, V.K., Buckley, S.A., Stanker, L.H., Kogut M.H. Prophylactic administration of immune lymphokine derived from T cells of Salmonella enteritidis-immune pigs: protection against Salmonella cholerasuis organ invasion and cecal colonization in weaned pigs. Advances in Experimental Medicine and Biology. 1999. v. 473. p. 299-308.

Genovese, L.L., Lowry, V.K., Genovese, K.J., Kogut, M.H. Longevity of augmented phagocytic activity of heterophils in neonatal chickens following administration of Salmonella enteritidis-immune lymphokines. Avian Pathology. 1999. v. 29. p. 117-122.

Lowry, V.K., G.I. Tellez, D.J. Nisbet, G. Garcia, O. Urquiza, L.H. Stanker, and M.H. Kogut. Efficacy of Salmonella enteritidis-immune lymphokines on horizontal transmission of S. arizonae in turkeys and S. gallinarum in chickens. International Journal of Food Microbiology 1999. v. 48. p. 139-148.

PROCEEDINGS/ABSTRACTS:

Bischoff, K.M., T.L. Crippen, M.H. Kogut, and D.J. Nisbet. 2000. The chicken Mim-1 gene product, p33, is a heterophil chemotactic factor found in *Salmonella enteritidis*-immune lymphokine. Annual Meeting of the American Society for Microbiology. May 2000, Los Angeles, CA.

Kogut, M.H., K.J. Genovese, and V.K. Lowry. 2000. Differential activation of signal transduction pathways mediating phagocytosis, oxidative burst, and degranulation by chicken heterophils in response to stimulation with opsonized *Salmonella enteritidis*. July 2000. VII Congress of the International Society for Developmental and Comparative Immunology. Cairns, Australia.

Kogut, M.H., V.K. Lowry, G.I. Tellez, G. Garcia, O. Urquiza, and D.J. Nisbet. 2000. Efficacy of *Salmonella enteritidis*-immune lymphokines on horizontal transmission of *S. arizonae* in turkeys and *S. gallinarum* in chickens. 2nd International Veterinary Vaccines and Diagnostic Conference, Oxford, UK. July 2000.

4.4

CRIS Title: Prevention and Control of *Salmonella* and other Enteropathogens in Poultry During Growout
CRIS: 6202-42000-011
Scientists: Kubena LF, Byrd JA, Ziprin RL, Hume ME
Location: Food and Feed Safety Unit, SPARC, College Station, TX
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Summary Project Aims:
Poultry meat products contaminated with Salmonella and Campylobacter bacteria are a major source of human food-borne illness. Our research focuses on the development of efficient and cost effective methods to prevent harmful bacteria from living in the gastrointestinal tract of live poultry. We are exploiting beneficial bacteria to provide protection against infection by harmful bacteria via a process called competitive exclusion. We are conducting research focused on the crop as a major source of bacterial colonization and ultimately meat contamination. We are evaluating critical control points such as feed withdrawal and transport to processing plants as opportunities for effective pathogen management. Research is also being conducted using compounds in the drinking water to reduce or control pathogens at critical times, and also dietary modifications in laying hen diets to manage Salmonella enteritidis. The work is partially targeted to small poultry farmers who will benefit economically by being able to produce microbiologically-safer poultry and eggs through use of relatively simple and cost effective methods.

Summary Accomplishments During Entire Project:
We have developed Salmonella control technology for poultry that is in the marketplace and that is used to treat millions of birds annually. The product, a defined competitive exclusion (CE) culture (PREEMPT**TM) was developed, patented, and licensed to our industry partner. In collaboration with land grant universities, other USDA agencies, and commercial producers, we developed a fluorescent marker technique to determine sites of carcass contamination in poultry processing plants. We showed that changes in the environment of the crop can significantly affect SE survival and influence the susceptibility to SE infections in live chickens. Our work on reducing gut colonization of poultry by pathogenic bacteria, and on reducing the incidence of Salmonella enteriditis in eggs, will have a major positive impact in protecting the consumer of poultry products from harmful bacteria.

Summary 2000 Accomplishments:
Cost-efficient ways to reduce Salmonella and Campylobacter in the crops of broilers prior to entry into the processing plant are needed. In collaboration with land grant universities, other USDA agencies, and our industry partners, we showed lactic acid to be effective in reducing pathogenic bacteria in broilers. When broilers were administered lactic acid in the drinking water during feed withdrawal just prior to slaughter, subsequent carcass contamination by Salmonella and Campylobacter was significantly reduced. These findings should offer an economically viable method of reducing poultry meat contamination by these important pathogens, and ultimately should result in safer poultry meat products reaching the consumer.

The laying hen industry is in need of effective methods to reduce Salmonella enteritidis (SE) contamination of eggs during forced resting. Chlorate added to drinking water before and during a forced resting period significantly reduced the incidence of egg contamination by SE. Certain dietary modifications during the forced resting period also reduced the incidence and degree of egg contamination by SE. These findings are important because they offer potentially viable methods of reducing or eliminating SE in eggs that will greatly enhance safety to the consumer.

Projected Research Accomplishments During Next 3 Years:
Determine the effects of feed withdrawal programs on gastrointestinal leakage after birds enter the processing plant. Improved delivery systems will be developed for PREEMPT**TM. The effects of lactic acid and chlorate in the drinking water, and of molt induction diets, on SE colonization/organ invasion in laying hens will be determined. Management programs that effectively decrease Salmonella will be evaluated in field trials. Pulse field gel electrophoresis and ribotyping will be utilized to expand our knowledge base of gut microbial ecology. Evaluation of PREEMPT**TM in turkeys will be initiated. Pathogen intervention strategies will be integrated into a single multi-faceted program for reduction of pathogenic bacteria in poultry. Work to improve CE efficacy will continue, and research on modified laying hen diets as affecting the microecology of the gut, alterations of the virulence of salmonellae, and SE egg contamination will be completed.

Technology Transfer:
Several commercial poultry producers have adopted the use of lactic acid in drinking water to reduce Salmonella contamination of broiler carcasses. This technology has been made available to other scientists and industry via publications in scientific journals. We do not forsee significant constraints to the more widespread acceptance of this technology by the poultry industry.

PUBLICATIONS:

Kwon, Y.M., Woodward, C.L., Corrier, D.E., Byrd, J.A., Pillai, S.D., Ricke, S.C. Recovery of a marker strain of Salmonella typhimurium in litter and aerosols from isolation rooms containing infected chickens. Journal of Environmental Science and Health. 2000. v. B35. p. 517-525.

Byrd, J.A., Corrier, D.E., Hume, M.E., Bailey, R.H., Stanker, L.H., Hargis, B.M. Effect of feed withdrawal on the incidence of Campylobacter in the crops and ceca of market age broiler chickens. Avian Diseases. 1999. v. 42. p. 802-806.

Corrier, D.E., Byrd, J.A., Hargis, B.M., Hume, M.E., Bailey, R.H., Stanker, L.H. Survival of Salmonella in the crop contents of market-age broilers during feed withdrawal. Avian Diseases. 1999. v. 43. p. 453-460.

Kubena, L.F., Harvey, R.B., Buckley, S.A., Bailey, R.H., Rottinghaus, G.E. Effects of long-term feeding of diets containing moniliformin, supplied by Fusarium fujikuroi culture material, and fumonisin supplied by Fusarium moniliforme culture material, to laying hens. Poultry Science. 1999. v. 78. p. 1499-1505.

Durant, J.A., Corrier, D.E., Byrd, J.A., Stanker, L.H., Ricke, S.C. Feed deprivation affects crop environment and modulates Salmonella enteritidis colonization and invasion of leghorn hens. Applied Environmental Microbiology. 1999. v. 65. p. 1915-1923.

Durant, J.A., Nisbet, D.J., Ricke, S.C. Response of selected poultry cecal probiotic bacteria and a primary poultry Salmonella typhimurium isolate grown with or without glucose in liquid batch culture. Journal of Environmental Science and Health. 2000. v. B35. p. 503-516.

Nisbet, D.J., Anderson, R.C., Corrier, D.E., Harvey, R.B., Stanker, L.H. Modeling the survivability of Salmonella typhimurium in the chicken cecae using an anaerobic continuous-culture of chicken cecal bacteria. Microbial Ecology in Health and Disease. 2000. v. 12. p. 42-47.

Young, C.R., Ziprin, R.L., Hume, M.E., Stanker, L.H. Dose response and organ invasion of day-of-hatch Leghorn chicks by different isolates of Campylobacter jejuni. Avian Diseases. 1999. v. 43. p. 763-767.

Ziprin, R.L., Young, C.R., Stanker, L.H., Hume, M.E., Konkel, M.E. The absence of cecal colonization of chicks by a mutant of Campylobacter jejuni not expressing bacterial fibronectin-binding protein. Avian Diseases. 1999. v. 43. p. 586-589.

PROCEEDINGS/ABSTRACTS:

Pillai, S.D., Hersheim, A., Obenhaus, A. and Byrd, J.A. Male specific coliphages in poultry litter and on chick box trayliners. Poultry Science. 2000. v. 79 (Suppl. 1): 4.

Hume, M.E. and Byrd, J.A. Genotypic patterns of Campylobacter isolated from market-age broiler crops at pre- and post-feed withdrawal and carcass rinses. Poultry Science. 2000. 79 (Suppl. 1): 5.

Kubena, L.F., Byrd, J.A., Anderson, R.C., Buckley, S.A., and Corrier, D.E. Effect of selected acids administered in drinking water on colonization of Leghorn hens by *Salmonella enteritidis* during forced molting. Poultry Science. 2000. v. 79 (Suppl. 1): 5.

Byrd, J.A., Sams, A.R., Caldwell, D.J., and Hargis, B.M. Effect of selected modified atmosphere packaging on Campylobacter survival in raw poultry. Poultry Science. 2000. 79 (Suppl. 1): 54.

Herron, K.L., Caldwell, D.Y., Byrd, J.A., Caldwell, D.J., McKenzie, K.S., and Hargis, B.M. Effect of drinking water administration of selected peracids on *Salmonella* crop contamination following preslaughter fee withdrawal. Poultry Science. 2000. 79 (Suppl. 1): 89.

Musgrove, M.T., Berrang, M.E., Byrd, J.A., and Stern, N.J. Detection of *Campylobacter* spp. in ceca and crops with and without enrichment. Poultry Science. 2000. 79 (Suppl. 1): 90.

Byrd, J.A., Herron, K.L., McReynolds, J.L., Caldwell, D.J., Hargis, B.M., and Kubena, L.H. Effect of lactic acid administration in the drinking water during preslaughter feed withdrawal on Salmonella and Campylobacter contamination of broilers. Poultry Science. 79 (Suppl. 1): 112.

4.8

CRIS Title: Control of Salmonella in Domestic Animals
CRIS: 6612-42000-019
Scientists: Bailey JS, Cox NA, Craven SE, Stern NJ
Location: Poultry Microbiological Safety Research Unit
Contact: 706-546-3356 (P); 706-546-3771 (F), jsbailey@ars.usda.gov

Summary Projects Aims

Control of Salmonella and other pathogens in poultry will require a total effort throughout production, transportation, processing, retail, and consumption. The significant reduction of these pathogens on the final processed carcass will be greatly enhanced by growing and delivering to the processing plant poultry with no or very little of these pathogens. We have identified the hatcheries and the early grow-out period as the most critical times for control of these pathogens. We are: 1) identifying and quantifying sources and spread of *Salmonella* and *Clostriduium perfringens* through all phases of broiler production; 2) developing on-farm control procedures to prevent colonization of chickens and/or turkeys with *Salmonella*.

Summary Accomplishments During Entire Project

We developed a unique and innovative process for making and delivering an effective bacterial culture of normal and healthy intestinal microflora, Mucosal Competitive Exclusion (MCE), which dramatically diminishes in the intestinal tract of chickens the presence of *Salmonella* and to a lesser extent *Campylobacter*, the two leading causes of foodborne human enteric disease. This positive affect, which carries through grow-out and processing expresses itself both in a reduction in contaminated carcasses and in fewer bacterial cells per carcass. In FDA sanctioned field trials, no *Salmonella* was detected on the final processed carcasses. [This technology is the only competitive exclusion technology in the world which has demonstrated effectiveness on the final processed carcass going to the consumer.] This commercial product Mucosal Starter Culture™ (MSC) nor any other competitive exclusion product is a magic bullet. However, MSC will play a significant role as part of an overall program (this program significantly influenced by the research of these nominees) to reduce *Salmonella* in poultry production. At an anticipated cost of less than $.02/bird, MSC will provide the poultry industry a cost effective tool to meet *Salmonella* criteria established by FSIS with a subsequent reduction in the 1 to 8 billion dollar impact of foodborne illnesses caused by *Salmonella* and *Campylobacter* on the U.S. economy.

We have identified critical control points for *Salmonella* in chicken production and through the multi-state epidemiology project and more local intensive studies are adding additional information which will allow a more detailed risk analysis. Initial studies showed that the hatchery and young chicks coming from the hatchery were the most important source of salmonellae in chicken production. Our research to identify most efficient chemical sanitizers for fertile hatching eggs and for the hatch cabinet environment along with other industry initiatives have resulted in a reduction of salmonellae positive hatchery samples of from greater than 75% in 1990 to less than 25% positive in 1996. Continued reduction of salmonellae in the hatchery combined with the use of MSC offers the poultry industry a realistic, cost effective opportunity to produce chickens with significantly reduced levels of salmonellae which meet FSIS standards.

In addition to the reduction of salmonellae, the MSC has also been shown to reduce necrotic enteritis caused by C. perfringens. Studies from around the world have consistently demonstrated that the use of the MSC leads to healthier birds that grow faster and eat less feed.

Summary 2000 Accomplishments

The previously completed national epidemiological study identified both the hatchery and the environment as contributing sources of salmonellae on processed chicken. In the current studies, we determined the relative contribution of the parent breeder stock compared to the grow-out environment on the numbers and specific types of salmonellae on the final processed chicken carcasses. The data gathered in these studies will allow us to build on the information from the national epidemiology study and to more precisely design multifaceted intervention strategies to be incorporated into the national intervention study which the National Chicken Council has endorsed to reduce the presence of salmonellae on chicken going to the consumer.

Genetic relatedness testing of C. perfringens isolates from multiple integrated chicken operations using an automated riboprinting procedure revealed a high degree of genetic diversity. Breeder flocks, hatchery samples, dust/dirt, flying insects, and transport cages were all identified as being involved with the transmission of C. perfringens to the final processed carcass. This data is the first ever reported about the movement of C. perfringens in poultry production systems and will be invaluable in helping design effective intervention strategies to reduce the presence of C. perfringens on processed chickens going to the consumer.

Published research findings have suggested that in ovo application of antibiotics, the most common of which is gentamicin, would prevent competitive exclusion cultures from effectively working in young chickens to reduce prevalence of salmonellae. A series of studies were completed where we demonstrated that commonly used concentrations of gentamicin applied to eggs in ovo did not adversely affect the performance of the mucosal competitve exclusion product, Mucosal Starter Culture. Since greater than 80% of the U.S. chicken industry uses in ovo antibiotics, this finding is critical to the potential adoption of competitive exclusion by the U.S. chicken industry.

Projected Research Accomplishments During Next 3 Years

During the next year we will gain final approval of the MSC competitive exclusion product for chickens. With the assistance of the U.S. poultry industry and the cooperation of the FDA, we will initiate a multi-state, multi-integrator on-farm intervention study which may serve as the template for industry adopted good production practices to reduce pathogens in broiler chickens. We will evaluate intervention strategies to reduce *C perfringens* in chicken production. We will evaluate different methods to more accurately monitor and trace salmonellae in poultry production systems.

Technology Transfer

The competitive exclusion product Mucosal Starter Culture (MSC) is being brought to commercial practice through a cooperative research and development agreement (CRADA) with the Continental Grain Company. The process for MSC has been issued a U.S. Patent (#5,451,400) and patents have

been issued in several foreign countries. Collaborative research studies have been established with 5 of the 7 largest commercial chicken producers and one of the largest turkey producers in the U.S.

PUBLICATIONS:

Bailey, J.S., Lyon, B.G., Lyon, C.E., Windham, W.R. The microbiological profile of chilled and frozen chicken. Journal of Food Protection. 2000. v. 63(9). p. 1228-1230.

Bailey, J.S., Stern, N.J., Cox, N.A. Commercial field trial evaluation of mucosal starter culture to reduce *Salmonella* incidence in processed broiler carcasses. Journal of Food Protection. 2000. v. 63(7). p. 867-870.

Berrang, M.E., Cox, N.A., Frank, J.F., Buhr, R.J., Bailey, J.S. Hatching egg sanitization for prevention or reduction of human enteropathogens: A review. Journal of Applied Poultry Research. v. 9. p. 279-284.

Craven, S.E., Stern, N.J., Cox, N.A., Bailey, J.S., Berrang, M.E. Cecal carriage of *Clostridium perfringens* in broiler chickens given Mucosal Starter Culture™. Avian Diseases. 1999. v. 43. p. 484-490.

PROCEEDINGS/ABSTRACTS:

Bailey, J.S. Controle de Salmonella im incubatorio (Control of Salmonella in Hatcheries). Conferencia Apinico de Ciencia E Tecnologia Avicolas. 2000. v. 1. p. 31-39.

Bailey, J.S. Pre-harvest food safety in the United States. Proceedings of XXI World's Poultry Congress. 2000. v. XXI. Paper # S2.2.01.

Bailey, J.S. Best opportunities for meeting present and emerging standards for microbial pathogens. National Meeting on Poultry Health and Processing. 1999. p. 77-83.

Bailey, J.S., Stern, N.J., Cray, P.J., Craven, S.E., Cox, N.A. A multi-state epidemiological investigation of sources and movement of *Salmonella* through integrated poultry operations. Proceedings of United States Animal Health Association. 1999. p. 194-198.

Bailey, J.S., Cosby, D.E., Stern, N.J., Cox, N.A. *Salmonella* and competitive exclusion in layers. Proceedings Southeastern Commercial Egg Producers Forum. 2000. p. 11-24.

Bailey, J.S., Cox, N.A., Stern, N.J., Craven, S.E. *Salmonella* prevalence rates from the breeder farm though the processing plant of an integrated poultry operation. Proceedings of XXI World's Poultry Congress. 2000. v. XXI. # P16.07.

Bailey, J.S., Stern, N.J., Cox, N.A. Food safety at the farm. Proceedings of Virginia Poultry Health and Management Seminar. 2000. p. 9.

Bailey, J.S. Food borne illnesses, fighting the battle from farm to fork. Proceedings of Southeastern Association of Clinical Microbiology. 1999. p. 46.

Bailey, J.S., Stern, N.J., Cox, N.A., Line, J.E. Preharvest strategies for reducing pathogen levels in animal production systems. Proceedings of Society of Industrial Microbiology. 2000. p. 42.

Bailey, J.S., Line, J.E. Effect of *in ovo* gentamicin on efficacy of mucosal competitive exclusion™ to control *Salmonella* in broiler chickens. Poultry Science. 2000. v. 79(S). p. 90.

Cox, N.A., Bailey, J.S., Cray, P.J., Stern, N.J., Berrang, M.E. Mucosal starter culture™ treatment to significantly reduce salmonellae in processed broiler carcasses and swine. World Health Organization Technical Report. 1999. p. 60-66.

Cox, N.A., Bailey, J.S., Stern, N.J. Use of mucosal starter culture™ to reduce salmonella during broiler production. Proceedings Midwest Poultry Federation. 1999. p. 85-89.

Craven, S.E., Stern, N.J., Bailey, J.S., Cox, N.A., Fedorka-Cray, P. Ribotyping for the differentiation of *Clostridium perfringens* isolated from poultry production and processing. 2000. Poultry Science. v. 79(S). p. 75.

Craven, S.E., Cox, N.A., Stern, N.J., Bailey, J.S. Prevalence of *Clostridium perfringens* in hatcheries of integrated poultry operations. Poultry Science. v. 79(S). p. 74.

Russell, S., Bailey, J.S. A new microbiological monitoring system that may be integrated into a HACCP program. Broiler Industry. 2000. v. p. 30-36.

4.12

CRIS Title: *Campylobacter* spp. Epidemiology, Methods Development and Interventions in Poultry.
CRIS: 6612-42000-031
Scientists: Stern NJ, Cox NA, Line JE, Hiett KL, Siragusa G
Location: Poultry Microbiological Safety Research Unit
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Summary Projects Aims:
A global human health concern, *Campylobacter* continues to be the leading bacterial cause of intestinal disease in the United States. Prevalence in fresh poultry meat is often greater than 80% and concentrations of the bacterium is routinely high. Effective interventions for poultry producers are lacking because we do not know where to apply such measures, as well as what measures need to be used. We are gathering data in poultry production to identify the most important sources, to create directed interventions, and to reduce the public exposure.

Summary Accomplishments During Entire Project
We coordinated with five of the nation's top ten broiler companies to conduct cooperative epidemiological studies. This was the first government-industry cooperative study to obtain data useful for developing quantitative risk assessment models in food animal production and should aid in subsequent intervention strategies. During the past five years we have created and patented agar media for the detection and quantitation of *Campylobacter* in poultry samples. Further, we have created a genetic subtyping system enabling us to track the organism through a number of defined ecosystems.

Summary 2000 Accomplishments
The most important source(s) of *Campylobacter* in broiler production remains controversial. We isolated and genetically characterized *Campylobacter* from commercial breeder flocks and their offspring broiler flocks, together with the corresponding processed carcasses. Our genetic evidence provided definitive proof that *Campylobacter* could be transmitted through bird generations via the fertile hatching egg. This finding could alter our approach in controlling the pathogen. Canadian and Icelandic SCA continued.

Projected Research Accomplishments During Next 3 Years
To describe how *Campylobacter* transmits through the hen reproductive tract into the fertile hatching egg. To create the required relations for exhaustive *Campylobacter* sampling from the relevant Icelandic samples. Initiate sampling of relevant poultry production sites for *Campylobacter* and be provided human isolates to assess importance of those poultry isolates. Conduct the genetic analysis of the numerous *Campylobacter* isolates we have gathered to delineate the importance of the various sources sampled. After the most important sources are identified, we shall begin to create innovative interventions that control those implicated sources. Test the novel intervention approaches we have developed in Iceland to determine whether poultry contamination may be controlled and human disease diminished. Apply successful interventions to the United States poultry industry.

Technology Transfer
We continue to provide intermittent *Campylobacter* methodology training to various members of the United States poultry industry and the USDA-FSIS. The epidemiologic studies are being conducted cooperatively with the United States poultry industry and, as the data become available, it is immediately incorporated into the industry best management practices.

PUBLICATIONS:

Buhr, R. J., Cox, N. A., Stern, N. J., and Wilson, J. L. Recovery of Campylobacter jejuni from segments of the reproductive tract of broiler breeder hens. Poultry Sci. 79:112. 2000.

Cox, N.A., M.E. Berrang, Stern, N.J. and Musgrove, M.T. Difficulty in Recovering Inoculated Campylobacter jejuni from Dry Poultry-Associated Samples. J. of Food Prot. Vol. 64, No. 2, 2001, Pages 000-000.

Craven, S. E., Stern, N.J., Line, J. E., Bailey, J. S., Cox, N. A., and Fedorka-Cray, P. J. Determination of the incidence of Salmonella spp., Campylobacter jejuni, and Clostridium perfringens in wild birds near broiler chicken houses by sampling intestinal droppings. Avian Dis. 44:715-720.2000.

Musgrove, M. T., Berrang, M. E., Byrd, J. A., and Stern, N. J. Detection of Campylobacter spp. in ceca and crops with and without enrichment. Poultry Sci 79 (Suppl.). 2000.

Stern, N.J., Hiett, K. L., Cox, N. A., Alfredsson, G. A., Kristinsson, K. G., and Line, J. E. Recent developments pertaining to Campylobacter. Irish Journal of Agricultural and Food Research 39:183-187.2000.

Stern, N.J., Patton, C. M., Doyle, M. P., Park, C. E., and McCardell, B. A. Campylobacter, In Vanderzant, C. and Splittstoesser, D. F. (eds.) Compendium of Methods for the Microbiological Examination of Foods., 4th ed, American Public Health Association, Washington, DC. 2001.

PROCEEDINGS/ABSTRACTS:

Buhr, R. J., Cox, N. A., Stern, N.J., and Wilson, J. L. Recovery of Campylobacter jejuni from segments of the reproductive tract of broiler breeder hens. Proc.Southern Poultry Science Association. Southern Poultry Science Association, Atlanta, GA, 2000.

Cox, N. A., Stern, N.J., Hiett, K. L., and Berrang, M. E. Campylobacter from Breeder to Broiler through the Fertile Egg. Summit III. Foodborne Pathogens in Poultry. p. 22. Watt Publishing, Atlanta, GA, 2000.

Cox, N. A., Stern, N. J., Bailey, J. S., and Craven, S. E. Prevalence of Campylobacter at several stages of an integrated commercial broiler operation. World Poultry Science . 2000.

Cox, N. A., Stern, N.J., Hiett, K. L., and Berrang, M. E. Egg transfers Campylobacter from breeders to broilers. 15:(9)World's Poultry. 6.2000.

Cox, N. A., Stern, N.J., Hiett, K. L., and Berrang, M. E. Passage of Campylobacter from breeder to broiler through the fertile egg. New England Conference on Poultry Health. 2000.

Craven, S. E., Stern, N.J., Line, J. E., Bailey, J. S., Cox, N. A., and Fedorka-Cray, P. J. Determination of the incidence of Salmonella spp., Campylobacter jejuni, and Clostridium perfringens in wild birds near broiler chicken houses by sampling intestinal droppings. Avian Dis. 44:715-720.2000.

Line, J. E., Stern, N.J., Bailey, J. S., and Cox, N. A. Saccharomyces boulardii treatment to diminish Campylobacter and Salmonella in Poultry. United States Pat.# 6,010,695. 19.2000.

Lowman, R., Paoli, G., Reierson, Lammerding, A., and Stern, N.J. Opportunities for process risk modeling and management of a closed-system foodborne epidemic in Iceland. Federal Food Safety and Nutrition Research Meeting. p. 61.,Ottawa, Canada, 2000.

Stern, N.J. Vertical transmission of Campylobacter. Proceedings of the Millennial Conference of the National Poultry Improvement Plan. Colorado Springs, CO. 2000.

Stern, N.J., Cox, N.A., Musgrove, M.T. and Park, C.M. Incidence and Levels of Campylobacter in Broilers After Exposure to Inoculated Seeder Birds. C16.45. Poultry Science Meeting, Montreal, Canada. 2000.

Stern, N.J., Bailey, J. S., Cray, P. J., Craven, S. E., and Cox, N. A. A Summary of a Major ARS Epidemiological Study to Determine the Flow of Campylobacter and Salmonella in Poultry Operations. Summit III. Foodborne Pathogens in Poultry. p.101. Watt Publishing, Atlanta, GA, 2000.

Stern, N.J. Tracking Campylobacter spp. in Poultry Operations and Humans. Convegno Annuale Società Italiana Patologia Aviare. 39:pp.14-16.2000. Forli, Italy

Stern, N.J. Selected Epidemiological Investigations Pertaining to Transmission of Campylobacter. Problems of Biological and Ecological Safety. pp. 348-349. 2000, Oblensk

Stern, N.J., Hiett, K. L., Cox, N. A., Bailey, J. S., and Craven, S. E. Strategies and Successes in Pathogen Control During Poultry Production and Processing. Proceedings at the "National Conference on Animal Production Food Safety." St. Louis, MO, 2000.

Stern N.J. and J.E. Line. Campylobacter, In Lund, B.W., Baird-Parker, T.C., and Gould, G.W. (eds.) The Microbiological Safety and Quality of Food. Aspen Publishers, Gaithersburg, MD. Volume II, pp.1040-1056. 2000.

Stern, N.J. Vertical transmission of Campylobacter. Proceedings of the Millennial Conference of the National Poultry Improvement Plan. Colorado Springs, CO. 2000.

4.16

CRIS Title: Pathogenesis, Detection, and Control of *Salmonella enteritidis* and Other Salmonellae in Chickens
CRIS: 6612-32000-017
Scientists: Gast RK, Petter JG, Holt PS, Mitchell BW, Swayne DE
Location: Southeast Poultry Research Laboratory, Athens, GA
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Summary Project Aims:
This project focuses on explaining the pathogenesis of *Salmonella enteritidis* (SE) infections in chickens and developing improved methods for prevention, detection, and control. Among the principal goals of the research are determining the processes and mechanisms by which SE infects chickens, spreads vertically and horizontally, and is deposited in eggs; assessing the influence of strain variations on the ability of SE to invade host organs and contaminate eggs; evaluating environmental factors that result in the emergence of virulent forms of SE from avirulent populations; developing more sensitive and specific diagnostic tests for identifying SE infections of chickens and for detecting SE contamination of eggs; developing and evaluating effective killed and live vaccines for controlling SE infections in chickens and associated egg contamination; and developing electrostatic space charging technology to diminish the airborne spread of SE throughout poultry hatching and housing facilities.

Summary Accomplishments During Entire Project:
Demonstrated that hens systemically infected with SE could produce internally contaminated eggs. Found that SE infections can be highly persistent in both chicks and hens and elicit long-lasting antibody titers. Developed and assessed the sensitivity and predictive value of diagnostic methods for detecting specific antibodies in serum and egg yolks from infected hens. Developed effective and practical bacteriological methods for consistently detecting very small numbers of SE contaminants in eggs. Developed and showed that killed vaccines reduced the susceptibility of laying hens to SE infection. Showed air movement transmitted SE infection. Determined that SE produced high molecular weight lipopolysaccharides (HMW LPS), but that other important pathogenic serotypes do not. Improved extraction and analytical methods for assessing structure of HMW LPS. Developed an epidemiological model for associating cell surface features of SE with the potential for egg contamination. Isolated the first strain of SE to exhibit classic signs of cellular communication by quorum-sensing. Described immunological and structural characteristics of biofilm formed by SE that has enhanced oral invasiveness and resistance to harsh environmental conditions. Demonstrated that some strains of SE become parenterally adapted. Patented a bacteriological medium that induces cellular differentiation and hyperflagellation by SE. Demonstrated that an electrostatic space charge system (ESCS) in egg hatching cabinets reduced airborne dust and food safety bacteria. The ESCS reduced airborne SE in an isolation room with caged layers by approximately 95%. The kill rate on airborne and surface SE at close range has been shown to be 98% or more. The ESCS has been patented and an exclusive license for poultry applications has been approved with BioIon, Inc. to manufacture and distribute the system.

Summary 2000 Accomplishments

In seven trials conducted in a commercial hatchery in North Georgia, an electrostatic space charge system (ESCS) reduced Enterobacteriaceae by an average of 94% and dust by an average of 77% compared to hydrogen peroxide treatment. This study demonstrated that an electrostatic space charge system can be significantly more effective than the commonly used hydrogen peroxide treatment for reducing the potential for airborne transmission of disease-causing organisms such as Enterobacteriaceae, and it will probably increase the interest of the poultry industry in adopting the electrostatic technology as a preferred treatment for all hatching cabinets.

More information is needed to understand why strains of SE vary in their ability to be orally invasive, because this is an early step in the infectious process that precedes egg contamination and it might be a critical control point that can be exploited to improve food safety. Different mutants in important virulence proteins of SE were constructed in a wildtype strain that had become parenterally adapted; they were used to challenge chicks by both the oral and subcutaneous routes. The results showed that some mutations increased oral invasiveness. The impact of this work is that it could help improve vaccines and our ability to reduce egg contamination by SE.

Because refrigeration of eggs has been suggested as a strategy to reduce opportunities for contaminated eggs to serve as a source of transmission of SE to humans, we sought to determine whether small numbers of SE cells could multiply to higher and more dangerous levels before refrigeration achieves internal egg temperatures capable of restricting further bacterial growth. We experimentally inoculated liquid egg components to examine SE multiplication in various locations during 3 days of incubation at different temperatures. Rapid and extensive SE multiplication often occurred at warm temperatures, especially when the bacteria had an opportunity for access to yolk nutrients and when contaminated eggs were incubated for several days before sampling. This demonstrates that SE deposited in or on the yolk can multiply rapidly if internal egg temperatures are not quickly lowered to growth-inhibiting levels.

Projected Research Accomplishments During Next 3 Years:

In 2001, we will immunize young growing birds with a purified invasion protein from *Salmonella* and assess its safety and efficacy for protecting against SE challenge. An additional focus of research efforts in this year will be to determine the relationship between the development of a specific humoral immune response (measured in both the blood and in egg yolks) and the production of eggs contaminated by SE. Our research with commercial hatcheries will continue in an effort to optimize the ECSC system for different hatcher types and for a small-scale breeder house, and then to determine its effect on dust and pathogen reduction, early mortality, and cecal contamination. We will also begin a study to determine the LD-50 dose for killing SE in biofilms. During 2002 we will challenge hens orally with mixtures of SE phenotypes that have different virulence properties to further assess the contribution of strain heterogeneity to egg contamination. Another series of studies will evaluate how the patterns of deposition and multiplication of SE isolates in egg contents affects the probable effectiveness of proposed standards for egg refrigeration. During this year we will study the effectiveness of the ESCS for pathogen reduction in egg rooms and we will expand pathogen reduction studies in hatcheries to include follow-up of chicks from ionizer treated hatching cabinets

into full-sized production houses. We will also determine the effectiveness of the ESCS for reducing pathogens and airborne disease transmission in a small-scale breeder house and investigate the mechanism by which ionization inactivates SE. Effectiveness of the ESCS for reduction of biofilms in large open areas will be determined. For 2003, we will screen soil and litter samples for factors that induce differentiation of *Salmonella*. We will also seek to develop improved models and methods for producing experimental SE contamination of eggs, detecting SE contaminants in eggs, and detecting specific antibodies in infected chickens. We will design an ESCS for full-sized breeder houses and test its effectiveness for pathogen and dust reduction.

Technology Transfer:
Improved methods for culturing eggs to detect SE contaminants have been incorporated into the testing protocols of federal trace-back programs and state egg quality assurance plans. A RADA was obtained from industry to apply knowledge of SE strain heterogeneity to improve vaccines. The ESCS system has been patented and licensed to BioIon, Inc. for dust and pathogen reduction in poultry areas. Commercial tests of the BioIon system have been conducted with four major poultry companies and are continuing with three of them. Numerous poultry companies throughout the world have expressed interest in the system. Dr. Mitchell received an ARS 1999 Superior Technology Transfer Award for his efforts with the system. A RADA was established in December, 1999 with BioIon for research and development of the ESCS system. During 1999, Dr. Mitchell participated in taped interviews for TV with Japan TV (October 7, 1999), and with Discoveries and Breakthroughs (April 20, 2000).

PUBLICATIONS:

Gast, R.K., Holt, P.S. Experimental horizontal transmission of *Salmonella enteritidis* strains (phage types 4, 8, and 13a) in chicks. Avian Diseases. 1999. v.43 (4). p.774-778.

Gast, R.K., Holt, P.S.. Influence of the level and location of contamination on the multiplication of *Salmonella enteritidis* at different storage temperatures in experimentally inoculated eggs. Poultry Science. 2000. v.79 (4). p.559-563.

Holt, P.S., Mitchell, B.W., Seo, K.H., Gast, R.K. Use of negative air ionization for reducing airborne levels of *Salmonella enterica* serovar enteritidis in a room containing infected caged layers. J. Appl. Poultry Res. 1999. v.8. p.440-446.

Mitchell, B.W., Stone, H.S. Electrostatic reduction system for reducing airborne dust and microorganisms. Patent Application Serial No. 09/122,850. Claims accepted Feb 25, 2000 by U.S. Patent Office.

PROCEEDINGS/ABSTRACTS:

Gast, R.K. *Salmonella enteritidis* in eggs and egg products: assessing and understanding the risks and responses. In Egg Nutrition and Biotechnology, Sim, J.M., Nakai, S., Guenter, W., eds., CABI Publishing, Wallingford, UK. 1999. p.431-440.

Gast, R.K. Deposition and multiplication of *Salmonella enteritidis* in eggs: reassessing the implications for applying refrigeration standards. Program of the Annual Meeting of the Salmonella Committee of the United States Animal Health Association. 1999. p.8 (Abstract).

Gast, R.K. Strategies and programs for controlling *Salmonella enteritidis* in poultry and eggs. Proceedings of 7th International Seminar on Poultry Production and Pathology, Chilean Association of Veterinary Poultry Specialists and the University of Southern Chile. 2000. p.29-35.

Gast, R.K. The relationship between the specific antibody response and the deposition of *Salmonella enteritidis* in eggs laid by experimentally infected hens. American Veterinary Medical Association Annual Convention Notes. 2000. p.695 (Abstract).

Gast, R.K., Holt, P.S.. Deposition of *Salmonella enteritidis* on and within egg yolks. Poultry Science. 2000. v.79 (Supplement 1). p.72 (Abstract).

Hamrita, T.K., Mitchell, B.W. Poultry environment and production control and optimization -- a summary of where we are and where we want to go. Transactions of the ASAE . 1999. v.42 (2). p.479-483.

Mitchell, B.W., Holt, P.S., Seo, K.H. Effectiveness of electrostatic space charge system for reducing dust in a caged layer room. AgEng Warwick. 2000. Abstracts Part 1. p.116-117 (Abstract).

Parker, C.T. Guard-Petter, J. The role of flagella in the pathogenesis of *Salmonella enteritidis* infection in chickens. 100th General Meeting, American Society for Microbiology. p.47 (Abstract).

Parker, C.T. Guard-Petter, J. The effect of O-antigen chain length on the pathogenesis of *Salmonella enterica* serovar Enteritidis infection in chickens. Meeting of the Southeastern Branch of American Society for Microbiology, Oct. 28-30, 1999. p.44 (Abstract).

4.20

CRIS Title: Epidemiology and Ecology of *Salmonella Enteritidis* in Commercial Poultry Flocks
CRIS: 6612-42000-022
Scientists: Petter JG, Holt PS, Gast RK, Swayne DS, Mitchell BW
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Summary Project Aims:
Identify the sources of introduction of *Salmonella enteritidis* (SE) and other salmonellae into commercial poultry flocks and the reservoirs where they persist in the poultry housing environment. Determine how SE spreads within and between commercial poultry flocks. Identify unique characteristics of *Salmonella* isolates, strains, serotypes, and phage types and apply this information to determine the epidemiological relationships between isolates from different sources.

Summary Accomplishments During Entire Project:
This research identified that it is possible to use the structure of the lipopolysaccharide molecule of SE and other *Salmonella* serotypes to conduct epidemiological investigations of how strain heterogeneity contributes to emerging patterns of salmonellosis in people and animals.

Summary 2000 Accomplishments:
Using cluster analysis of sugars extracted from bacteria, we have determined that SE exhibits much greater diversity than does *S. typhimurium* in regards to the type of major cell surface carbohydrate it produces. Collaborative research with the Ministries of Agriculture of Food and Fisheries (MAFF) in the United Kingdom also determined that sugar cluster analysis is also separating Salmonella enteritidis according to genetic diversity and source of strain. The outcome of this research is that it has provided improved epidemiological methods for monitoring emergence of virulent SE and other salmonellae that have enhanced potential to threaten the safety of the food supply.

Projected Research Accomplishments During Next 3 Years:
During each of the next 3 years, approximately 25 strains of *Salmonella* will be characterized using gas liquid chromatography to learn more about how *Salmonella* serotypes differ from each other. This CRIS is to become part of CRIS 6612-32000-017.

Technology Transfer:
Information and data was provided to the Food and Drug Administration and the Egg Nutrition Council about improved methods for analyzing *Salmonella* population biology. This information was in part responsible for procurement of a two year NRICGP grant (1998-2000) and a CRADA with industry to improve vaccines. Also, other scientists and collaborators in The United Kingdom were informed of these results during a seminar tour in England entitled "How *Salmonella enteritidis* differs from *Salmonella typhimurium*: a defined role for high molecular weight lipopolysaccharide in egg contamination." This seminar was presented at the Department of pathology at Cambridge University, Veterinary Laboratory Agency at Weybridge, Public Health Laboratory Service at Exeter, and the Institute of Pharmaceutical Sciences at Nottingham University.

PUBLICATIONS:

Potera, C. Pathogen uses diffusible signal to form biofilms and contaminate eggs. ASM News. Apr 2000. V. 66, p. 195-197.

PROCEEDINGS/ABSTRACTS:

Parker, C.T., B. Harmon, J. Guard-Petter. Reproductive tract pathology in hens from challenge with *Salmonella enteritidis* and a wzz mutant that lacks HMW LPS. 100th General Meeting of the American Society for Microbiology. Abstract p. 120.

Petter, J. G. Chemical characterization of *Salmonella* serotype and its use in epidemiological investigations. Proceedings, Workshop on Epidemiological Methods and Approaches for Food Safety. October 18-19, 2000. In Press.

4.22

CRIS Title: Disease Related Problems of Poultry Production and Processing
CRIS: 6226-42000-005
Scientists: Huff GR, Huff WE, Donoghue AM.
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Summary Project Aims:
Turkey osteomyelitis complex (TOC), affecting up to 13.7 million turkeys yearly, is a condition defined by the USDA Food Safety Inspection Service to include processed turkey carcasses which contain lesions including osteomyelitis, synovitis/arthritis, and soft-tissue abscesses. The primary bacterial species isolated from these lesions is *Staphylococcus aureus* which has the potential of producing toxins of food safety significance. These lesions are associated with the presence of a green liver and are currently detected by cutting the joints, bones, and muscles of processed turkeys which have a green liver. This research is resolving the problem by determining the cause of the disease and means of prevention.

This CRIS received additional funding and a research project is being planned to address the animal and human health concerns with antibiotic resistant organisms. The emergence of pathogenic bacteria resistant to antibiotics is a serious and world wide animal and human health concern. This global problem has greatly affected poultry production practices, and has provided an immediate need for alternatives to the use of antibiotics in poultry production. This research directly addresses that issue as methods are being developed that improve innate resistance to bacterial infections. It is important that alternatives to antibiotic therapy of animal and human diseases be pursued. Bacteriophage are being isolated and tested for their efficacy to target food-borne bacteria and to treat or prevent bacterial infections.

Summary Accomplishments During Entire Project:
The major accomplishment of this project has been the development of an experimental model for reproducing the lesions of turkey osteomyelitis complex using dexamethasone injection. This model has enabled us to design experiments to test the effectiveness of various immunomodulators for their ability to prevent not only TOC but also chronic respiratory infection with opportunistic pathogens such as *Escherichia coli* and *Staphylococcus aureus* which can have impact on food safety. This model has contributed substantially to our hypothesis that TOC is due to stress related immunosuppression, and that genetic selection may enable producers to select for birds with a more appropriate response to the stresses inherent in modern turkey production.

Summary 2000 Accomplishments:
Turkey osteomyelitis complex (TOC) results in potentially dangerous bacterial infections in turkey carcasses. We have developed an experimental model which suggests that stress-related immunosuppression results in susceptibility to these infections and allows us to test immunomodulators and management procedures for their ability to protect turkeys from the effects of severe stress. We have demonstrated that treatment of drinking water with vitamin E and salicylic acid can decrease mortality and *Escherichia coli* infection in this model. Further work with dosage

optimization may lead to an effective method for reducing the impact of stress on TOC as well as contamination of animal carcasses with bacteria of food safety importance.

Our experimental model for reproducing turkey osteomyelitis complex has suggested that genetic differences in the response to stress are involved in susceptibility to these infections. In collaboration with Dr. Nick Anthony, University of Arkansas, we have discovered that Coturnix quail which were divergently selected for increased early growth rate, have increased fearfulness levels as measured by behavior in a t-maze. We have shown that this fearfulness is positively correlated with a decrease in resistance to *Escherichia coli* infection in stressed birds. Extrapolation of these findings to chickens and turkeys may lead to selection measures for individuals capable of better growth and greater resistance to infection with bacteria of food safety importance under commercial management conditions.

This project has specific outreach activities which support small farms. Outreach to members of a local growers association has led to the realization that the loss of product resulting from this problem is sometimes managed by increasing bird density. This can result in even more disease due to stress and more loss by the individual grower. This disease problem has put many small farmers at risk. We are providing support and consultation to small farmers experiencing loss due to high TOC incidence and we are attempting to provide long term solutions to this problem.

Projected Research Accomplishments During Next 3 years:
We will redirect the focus of this project toward the study of the impact of stress on the pathogenesis of bacteria of food safety importance. Specifically, studies of the pathogenesis of *Listeria monocytogenes* are being planned. Studies of the links between genetics, behavior, and disease resistance will be continued. Optimization of the dosages for treatment of stressed birds with vitamin E and salicylic acid will be completed. Work will be initiated to isolate bacteriophage to targeted bacteria.

We will continue to test various nutritional immunomodulators for their ability to prevent bacterial infections of food safety importance and decrease the industry's reliance on antibiotics. This work will result in improvements in the general health and stress resistance of poultry resulting in both decreased losses due to disease and less bacterial contamination of carcasses. We will continue to explore the genetic potential for stress resistance in poultry. Work will continue on the isolation of bacteriophage to organisms important to poultry product safety and poultry health, and their efficacy to improve product safety and improve poultry health will be determined.

We will continue research to decrease the levels of human pathogens in poultry products by improving the bird's response to stress and to provide alternatives to the use of antibiotics in poultry production, which is an important and immediate goal of a recent FDA/CDC/USDA Monitoring Program. Work will continue on the evaluation of the efficacy of bacteriophage to improve poultry product safety and health.

Technology Transfer:
A report describing preliminary studies of the effects of vitamin E and salicylic acid has been forwarded to the producer. The use of these products by the industry will require additional studies of both dosage and safety. The use of bacteriophage as a tool to improve poultry product safety and poultry health should begin to be presented to the scientific community within the next couple of years. The adoption of this technology by the poultry industry will depend on its efficacy and cost, which will not be apparent for several years.

PUBLICATIONS:

Huff, G.R., Huff, W.E., Balog, J.M., Rath, N.C. The effect of vitamin D_3 on resistance to stress-related infection in an experimental model of turkey osteomyelitis complex. Poultry Science. 2000. v.79(5). p.672-679.

Huff, G.R., Huff, W.E., Rath, N.C., Balog, J.M. Turkey osteomyelitis complex. Poultry Science. 2000. v.79(7) p.1050-1056.

PROCEEDINGS/ABSTRACTS:

Presentation to the National Turkey Federation, Turkey Health Symposium: Stress-induced Immunosuppression: A Link Between Respiratory Infection and Turkey Osteomyelitis Complex. Orlando, FL. Jan. 7, 2000.

CRIS Title: Biosensor Processes for Detecting Pathogenic Bacteria in Foods
CRIS: 1935-42000-040
Scientists: Tu S-I, Irwin P, Yu L, Brewster J
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Summary Project Aims:

The presence of pathogenic bacteria at any stage of food production, processing, and distribution must be quickly determined to allow proper treatments prior to consumption by the general public. Detection and quantification of pathogenic microorganisms in foods is vital for ensuring a safe food supply. There is a need for rapid, sensitive, and specific tests which researchers, farmers, processors, and retailers can use to verify that foods are safe to consume. Effective tests must meet a number of criteria: speed is critical since modern processing and distribution systems operate rapidly; high sensitivity is desirable since an infectious dose may be as little as one organism; selective detection is required because pathogenic bacteria comprise a small fraction of an otherwise benign population of microorganisms. Thus, we conduct research to develop new, or modify existing technologies to increase the speed, specificity and sensitivity of detecting food-borne pathogens to meet the needs of food producers/processors and regulatory agencies.

Summary Accomplishments During Entire Project

Since the inception of our current CRIS in 1996, we have adopted the use of IMBs to separate and concentrate pathogens for detection by a variety of biosensors. Some examples described below, could detect *E. coli* O157:H7 at a level of 1 CFU g^{-1} (beef hamburger) after enrichment at 37 ^{o}C for 5–6 hrs. (1) We have developed an immunomagnetic electro-chemiluminescent (IM-ECL) method to detect *Escherichia coli* O157 in ground beef, using commercially available analytical instrument and have also developed a confirmatory method based on immunomagnetic cell capture (IMCS) with plating on chromogenic media. We have collaborated with a government inspection agency to adapt the method to the procedures used in their laboratories. (2) A government inspection agency has incorporated our approaches for immunomagnetic cell separation and plating on Rainbow® agar O157 media into their official methods, and although the IM-ECL assay is 10 to 100 times more sensitive than the test they currently use. However, they have not adopted this presumptive assay because of the physical manipulations required for heat killing and carousel loading. The inspection agency has also requested that the IM-ECL assay be put in a 96 well format which can then be incorporated into a robotic system. (3) We have used IMBs to capture *E. coli* O157:H7, treated the organism with a fluorescent nucleic acid stain (DAPI), and used fluorescent microscopic imaging for detection. In this work, we applied a magnet to concentrate and align the IMB on the microscope slides for faster counting of fluorescent area and enumerated the bacteria by the use of an automated 2-dimensional microscope stage and CCD camera. This process should reduce the enumeration time and eliminate the operator fatigue associated with manual microscopic counting. (4) We have devised a new procedure to determine the viability of detected *E. coli* O157:H7. In this approach, the energetic status of the bacteria was adjusted by the addition of glucose, a carbon nutrient source, and carbonyl cyanide meta-chlorophenyl hydrazone (CCCP), a membrane protonophore. The addition of glucose slightly increased the ATP content of the bacteria.

On the other hand, CCCP depleted the ATP content. None of the glucose and CCCP effects could be detected with heat-killed and -ray irradiated *E. coli* O157:H7. Thus, immunomagnetic capture of the *E. coli* followed by described tests would confirm the presence of viable *E. coli* O157:H7. Bioluminescence determination of ATP has been widely adopted by food industry to monitor sanitary conditions. The approach developed by us can increase the value of this screening technique.

To minimize the need of enrichment, methods that can concentrate small quantity of targeted pathogens in samples with large volume, must be developed. To meet this goal, we have developed micro-immunoaffinity columns for capture and concentration of bacteria from food samples. Initial experiments used porous, low-density polyacrylamide particles to capture *Salmonella enteriditis*. While effective in capture, these particles required centrifugation to separate from the sample. Attempts to visualize or detect bacteria bound to the particles with fluorescent or enzyme-conjugated antibody was difficult due to high background from antibody trapped in the pores of the particles. We have now prepared columns using solid, high-density glass beads that exhibit low background binding and settle rapidly to allow simple separation of the particles from the sample. These columns have been used to capture *E. coli* O157:H7 and *Campylobacter coli* with >90% efficiency

Summary 2000 Accomplishments

Established Theory and Methodology for High Precision Enumeration of Bacteria: Enumeration of bacteria is an essential part of any research program on detection, but current methods of enumeration have serious deficiencies in terms of preparation time, throughput, and accuracy. We recently developed a new technique, the micro-growth method, for automated, accurate, high-throughput enumeration. In the past year, we have tested a range of microorganisms to include *Salmonella, E. coli* O157:H7, *Campylobacter coli*, and *Lactobacilli*. We have also developed and experimentally confirmed the theory for self-calibration using serial dilutions of an unknown sample. This allows the micro-growth method to be used for absolute enumeration without standards, and removes the only significant limitation of the methodology.

Modeling the Capture of Pathogenic Bacteria by Immunomagnetic Beads (IMB): IMBs capture targeted bacteria using the specific antibody coated on the bead surface. The captured bacteria can then be separated from other components on/in the food matrix with a magnet. However, there is no quantitative information on the conditions to achieve maximum capture. We have developed a systematic approach to mathematically model the capture of any (target or non-target) bacterial species with any IMB. This model allows us to predict the quantity of IMB and mixing time needed to achieve desired capture and thus develop standard procedures for using IMB techniques in high throughput laboratories.

We determined the cause of the apparent loss of bacterial activity during magnetic separation (MS) of IMBs: There is a loss of approximately 5-15% of the cellular activity associated with IMB-captured bacteria per MS. Our research has shown that this value varies mainly as a function of the number of cells captured per IMB and is not due to the death of captured cells. These data argue that

the loss is related to an increased buoyancy of the bacteria-IMB complex. This work is significant in as much as it indicates that repeated MS steps (e.g., as in washing) should be avoided and that maximum efficiency is attained when the ratio of IMBs to cells is much greater than one.

A rapid, sensitive and 96-well micro plate reade-based method for detecting *Escherichia coli* O157:H7 in beef hamburgers was developed: The 96-well micro plate reader is widely used in food microbiology laboratories for applications such as enzyme linked immunosorbent assay (ELISA) of bacterial cultures after overnight enrichment. To maximize the practical value of the 96-well apparatus, we have incorporated immunomagnetic bead capture and separation of *E. coli* O157:H7 from artificially spiked beef hamburger and an alkaline phosphatase linked ELISA method for detection. We found that this approach detected the presence of 1 cell of the E. coli per gram of hamburger after a four-hour incubation. This result demonstrated that proper modification of testing procedures could provide rapid and sensitive pathogen detection using existing laboratory apparatus.

Current practice of bacterial analysis requires shipping of large chucks of meat from remote processing locations to central laboratories. The practice is costly and may introduce additional contaminations. In the process of developing new detection method for *E. coli* O157:H7 in beef hamburger using a device called Light addressable potentiometric sensor (LAPS), we used streptavidin coated magnetic beads conjugated with proper antibody to capture the *E. coli*. The beads and captured bacteria were then locked on a biotin-coated membrane, exhibited a relatively long stability toward LAPS detection at refrigerated temperature. We have now improved the stability to about three days at room temperature by the use of smaller magnetic beads. This information is useful for food processing institutions relying on external bacterial testing support.

Developing time-resolved -fluorescence assay for Pathogenic Bacteria Detection: Fluorescence detection of pathogens is often compromised by similar fluorescence associated with other components in foods. We have utilized a method called time-resolved-fluorescence (TRF) approach in which targeted bacteria were tagged with antibody containing europium, a rare-earth metal ion that maintained the ability to emit specific fluorescence long after interfering fluorescence faded away. Thus, by delaying the start of measurement we could specifically correlate fluorescence intensity to the quantity of pathogens in foods. With this fluorescence measurement method and immunomagnetic capture approach, we were able to detect about 10 *E. coli* O157:H7 spiked in one milliliter of apple cider after a four-hour enrichment and about 1 cell of *E. coli* O157:H7 spiked in 1 gram of beef hamburger after four and half hour enrichment.

Improving the process safety of the immunomagnetic electro-chemiluminescent method for *E. coli* O157:H7 detection: The immunomagnetic electro-chemiluminescent method we developed to detect E. coli 0157:H7 in food required heat-killing and filtering steps to alleviate any concern about potential aerosols generated during a mixing step and eliminate large particles that could block the fluidics system of the detection instrument. These steps are labor intensive and time consuming, so we examined various chemical techniques to kill the cells and ultimately eliminated the heat-killing step by incubating the cells in a reagent that disrupts the cell membrane. The disruption of the cell membrane releases additional cell membrane associated antigen which increases the sensitivity of

the assay and solubilizing the membrane eliminates the need to filter the sample. Eliminating the heat-killing and filtering steps allows robotics to be used to automate the assay thereby reducing cost and increasing throughput.

Projected Research Accomplishments During Next 3 Years

To develop magnetic columns using immunomagnetic beads as column packings to rapidly concentrate targeted food pathogens in food systems. The milestones are: to complete characterizing the interactions between of magnetic column materials and immunomagnetic beads; to complete the design of automatic control of the magnetic column and application to pure bacterial cultures; and to test the assembly in food systems.

To automate our developed detection processes to use 96-well format. The milestones are: to complete the reagent injection sequence study on the confirmation test of E. coli O157:H7 using ATP luminescence measurement; to complete the integration of sequence study to commercially available 96-well plate reader, and to expand the automation work to other pathogenic bacteria.

Technology Transfer

We worked with a government inspection agency to implement the assay its laboratories. The procedure for the assay was briefly adopted and was then put on-hold for certain laboratory safety issues. We are currently addressing these concerns. Because of our development of the assay for *E. coli*, our CRADA partner has formed a business division to develop and market kits to detect enteric pathogens, non-enteric pathogens (*Listeria, Salmonella, Campylobacter*, etc.) and other organisms such as parasites, mycotoxins, and mycoplasma. They have completed the conversion of the laboratory assay we developed for *E. coli* O157 into testing kits and are being evaluated by a few major fast food chains. Our partner has also redesigned the assay into a 96 well format high-throughput instrument. We formed a collaborative arrangement with an instrument company to develop procedures for detecting pathogenic bacteria in foods. The methodology included the applications of time-resolved–fluorescence measurements. Because the non-specific background fluorescence was minimized, the developed procedure would exhibit very low false-positive measurement and would also allow multiplex measurement of a single sample. We anticipate this collaboration would lead to the development of highly desirable and automated pathogen detection procedure for food industry and inspection laboratories.

PUBLICATIONS:

Fratamico, P., Crawford, C.G. *Escherichia coli* O157: Detection by commercial immunomagnetic particle-based assays. In Encyclopedia of Food Microbiology, Ed. Richard Robinson, Carl Batt, Pradip Patel. 1999. V. 1, p. 654-663, Academic Press.

Irwin, P.L., Brouillette, J.N., Germann, M., Hicks, K.B., Kurantz, M., Damert, W. Calculation of immobilized enzyme reaction progress curves from nested ordered-sequential rate expressions. Enzyme and Microbial Technology. 1999. V. 24., p. 675-686.

Irwin, P.L., Brouillette, J.N., Giampa, A., Hicks, K.B., Gehring, A., Tu, S. Cyclomaltoheptaose (b-cyclodextrin) inclusion complex formation with chlorogenic acid: hydration enthalpy, the solvent entropy (hydrophobic) effect, and enthalpy-entropy compensation. Carbohydrate Research. 1999. V. 332, p. 67-76.

Mazenko ,R.S., Riders, F., Brewster, J.D. Filtration capture immunoassay for bacteria: optimization and potential for urine analysis. Journal of Microbiological Methods. 1999. V. 36 p. 157-165.

Shen, S., Taylor, R.W., Tu, S. Equilibrium and spectroscopic studies of lead retention in smectite. Communications of Soil Science and Plant Analysis. 1999. V. 30, p. 2711-2730.

Tu, S. Patterson, D., Uknalis, J., Irwin, P. Detection of *Escherichia coli* 0157:H7 using immunomagnetic capture and luciferin-luciferase ATP measurement. Food Research Internationa. 2000. V. 33, p. 375-380.

Tu, S., Uknalis, J., Irwin, P., Yu, L.S.L. The use of strepavidin coated magnetic beads for detecting pathogenic bacteria by a light addressable potentiometric sensors (LAPS). Journal of Rapid Methods and Automation in Microbiology. 2000. V. 8(2). p. 95-109.

Yu, L.S.L., Bolton, D., Laubach, C., Kline, P., Oser, A., Palumbo, S. A. Effect of dehairing operations on microbiological quality of swine carcasses. Journal of Food Protection. 1999. V. 62(12), p. 1478-1481.

Yu, L.S.L., Palumbo, S.A. Enumeration of Aeromonas for verification of hygienic adequacy of swine carcass dressing processes. Journal of Food Safety. 2000. V. 20(1), p. 43-52.

5.6

CRIS Title: New Technology and Systems to Detect and Prevent Chemical and Microbial Food Contaminants
CRIS: 1935-42000-035
Scientists: Tu S-I, Linton R
Location: Eastern Regional Research Center, Wyndmoor, PA, and Purdue University
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Summary Project Aims:
The Food Safety Engineering Project (FSEP) is a joint effort between the USDA Agricultural Research Service (ARS) and the School of Agriculture at Purdue University. Established in 1999, the mission of the ARS-FSEP effort is to develop new knowledge, technologies, and systems to prevent chemical and microbial contamination of foods. Project research focuses on four objectives: developing diagnostic tools for rapid identification of biological and chemical foodborne contaminants; developing models to predict and track foodborne contaminants; identifying, designing and evaluating alternative processing, handling, packaging, transport, and storage systems to minimize and/or reduce food contaminants; and, developing technology transfer of information and knowledge related to food safety for the food industry, government agencies, academia, and the public.

The Competitive Research Funding Program established in 1999 funded several new multi-disciplinary research projects in FY2000: 1. Light scattering sensory method for rapid assessment of foodborne bacterial contaminants; 2. A virtual sensor for post-package pasteurization of RTE bologna; and 3. Rapid detection of total PCBs and toxicity equivalence quotient (TEQ) in fish tissue from Indiana waters and use of a novel device to predict contaminant load in fish. The FY-1999 funded project "Biosensor-based approaches for rapid and sensitive detection of *Listeria monocytogenes*" was expanded and additional funding was directed.

Summary Accomplishments During Entire Project:
Much of the research effort has been designed to assemble biosensor probes, develop suitable buffers and reagents, preparation and purification of antibodies to be used as a ligand, and optimize experimental conditions for biosensor based detection of *Listeria*. Preliminary data indicates an encouraging future showing that certain biosensor probes can be used to measure *Listeria* interactions with animal cells. However, the technologies require many refinements so that the small number of bacterial cells in foods can be detected with relative ease.

Summary 2000 Accomplishments:
Research with a tetra-polar impedance analyzer and fluorescence based assays with animal cells was conducted to detect pure cultures of *L. monocytogenes*. Cells can be detected in 1 hour, a marked improvement over previous non-biosensors. A multi-disciplinary team of researchers including engineers, chemists, and food scientists has been formed to develop a biosensor chip for detection of live and dead *L. monocytogenes*. Initial studies show that complex impedance (electronic measurement) was measured for a high concentration of cells put through the biosensor chip

containing an antibody-based system of detection. It is hoped that this method can be developed to detect live and dead cells at low levels, and can be cost effective for use on ready-to-eat foods.

Research is being done to detect *Fusarium* molds using an enzyme-linked immunosorbant assay (ELISA) and the polymerase chain reaction (PCR). Antibodies to *Fusarium* proteins are currently being produced in rabbits but the titers are not high enough to produce ELISA; however, PCR primer sets have been developed using ribosomal DNA and specific genes involved in mycotoxin biosynthesis. Three PCR primer sets were developed in Dr. Woloshuk's lab that detect *Fusarium* species (*F. graminearum, F. moniliforme, F. culmorum, F. sporotrichoides)* that produce trichothecenes and fumonisins.

An epidemiological study was done to determine S*almonella* infections and their relationship in poultry and egg products. Demonstration of the usefulness of certain molecular epidemiological tools in the establishment of epidemiological links between human infection with *Salmonella* and poultry products as potential sources was established.

Two novel non-thermal methods for reduction of *E. coli* O157:H7 on produce were evaluated: washing with ozonated or ClO_2 water; and, gaseous treatment with ozone or ClO_2. Under certain conditions, up to a 2.5 log reduction was obtained during the study carried out on lettuce and baby carrots for these treatments. When these methods are optimized we believe a 3-log reduction of pathogens can be obtained.

Projected Research Accomplishments During Next 3 Years:
The primary goal of the *Listeria* biosensor project is to engineer a biosystem to rapidly detect *L. monocytogenes* and other pathogens in foods. The biosystem being developed by our multi-disciplinary team combines protein chemistry with a computer chip at a micron scale. The result is anticipated to be a postage stamp size biosensor that will detect the presence of *L. monocytogenes* in a matter of minutes. This biosystem will have the capability to be coupled to a hand held device capable of reading and interpreting the signal from the chip, and thereby facilitate rapid, in-plant detection of a pathogenic organism. Such a configuration will enable rapid transmission of the results to a computer and ultimately through the Internet to remote locations, as needed. This biosystem is based on the concept of binding a protein (antibody) on electrically conductive surfaces placed on a non-conducting surface (such as silicon dioxide or plastic) of a microchip. The protein is chosen so that it will selectively bind with the pathogenic organism, if present, in a liquid sample passed over the chip. The goal is also to detect binding, and therefore the presence of a pathogenic organism, by a change in an electronic signature when the antibody on the surface of the chip binds with its antigen (a protein on the surface of the cell). Electronic detection is intended to supplant the more expensive optical methods that are currently available and in use (including in our laboratories). The biosystem will incorporate bioseparations technology in order to condition the sample by rapidly removing potential interfering compounds (including other proteins) before the sample is presented to the chip. The quantities of sample that will need to be processed are expected to be small since the volume of the channels on the chip are on the order of microliters or less. The specific objectives are: 1. characterize binding of polyclonal and monoclonal antibodies with *Listeria*

monocytogenes cells at silicon dioxide/platinum surfaces that will be representative of microchip based sensing systems; 2. carry out fundamental research on detecting the binding and state of cells (living or dead) using electrically based sensing on a micro-chip; and 3. develop methods for sampling, cell concentration, and sample delivery for a microchip-based system.

A second goal of the *Listeria* biosensor project will be to isolate and concentrate bacteria from food samples and subsequently detect them with (a) fiber optic probe, (b) tetra-polar impedance analyzer and (c) fluorescence assay. Also, ability of the biosensors to detect a low numbers (10-100) of bacterial cells will be critically examined.

For *Fusarium* detection, the project will result in the development of rapid assays to detect the presence of *Fusarium* species in grains and foods. One assay will be based on the production of antibodies to *Fusarium* species and the development of an ELISA to be used as a rapid screening tool (estimated as < 30 min) to determine if *Fusarium* species are present in grains to be stored or to be used for food processing. This information could help the grain storage operator to alter conditions to prevent the growth of *Fusarium* species and the subsequent mycotoxin production. It could also be used by food processors to divert grain from some processing where *Fusarium* species could grow and produce mycotoxins. The other, PCR assay could be used to determine either the general presence of *Fusarium* species or which individual *Fusarium* species are present because it is based on the genes for the mycotoxin production. Antibodies will be successfully produced and the ELISA will be developed for the detection of *Fusarium* species. The PCR primers will be evaluated to determine their specificity for detecting only *Fusarium* species and not other mold genera. Studies on the method for extraction of *Fusarium* DNA from grains and foods, plus the evaluation of whether enrichment techniques are needed will be done. A simple protocol for detecting toxigenic *Fusarium* species in foods will be developed. For the project involving non-thermal inactivation of pathogens on fresh produce, we will focus on washing fresh produce using ClO_2, ozone and plant extracts inoculated with pathogenic micro-organisms, then coupling these technologies with high pressure and mild heat treatment.

Technology Transfer:
Most of theses studies are to be completed in approximately 2 years. The developed technologies will be evaluated in local industries for their efficiency in commercial settings. Once the project has reached completion stage, the techniques that produce more promising results will be tested at the industrial scale.

PROCEEDINGS/ABSTRACTS:

Bashir, R. Gomez, A. Sarikaya, M. R. Ladisch, J. Sturgis, J. P. Robinson, A. Bhunia, "Towards a Protein Biochip: Micro-scale Detection of Biological Species in a Microfluidic Chip, NIH Symposium on Bio-Nanotechnology, Bethesda, MD., June 25-27, 2000.

Tan. R. Saeed*. A.M. Koons. C., Barreett. B. Teclaw. R.F., Bixler. D. Changing Trends In Human *Salmonella* Enteritidis infections in Indiana between 1992-1998. 103th Annual Meeting of the United States Animal Health Association. Committee on Public Health and Environmental Quality October 11, 1999. San Diego, California

Tan. R. Saeed*. A.M. Koons. C., Barreett. B. Teclaw. R.F., Bixler. D. Changing Trends In Human *Salmonella* Enteritidis infections in Indiana between 1992-1998. International Conference on Emerging Infectious Diseases. CDC. Atlanta. GA, July 16-19. 2000.

5.10

CRIS Title: Development of Methods and Strategies to Improve the Microbiological Safety of Aquaculture Products
CRIS: 1935-42000-036
Scientists: Richards GP, Kingsley D
Location: Microbial Food Safety Research Unit, Delaware State University, Dover, DE
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Summary Project Aims:
Hepatitis A virus (HAV) and Norwalk-like viruses (NLVs) are enteric viruses transmitted by the fecal-oral route. The Centers for Disease Control and Prevention recently estimated the annual incidence of HAV and NLVs in the United States at 83.4 thousand and 23 million cases, respectively. The NLVs are reportedly the leading cause of food-borne illness in the U.S. and are responsible for 7% of the food-related deaths. Water and person-to-person transmission may be responsible for the majority of illnesses from HAV. About 40% (about 9.2 million cases annually) of NLV illnesses are reportedly food-borne. Another food-borne pathogen linked almost exclusively to the consumption of molluscan shellfish, is a virulent form of *Vibrio vulnificus*, an indigenous marine bacterium which infects considerably fewer shellfish consumers, but has an incredibly high case fatality rate of about 50%. These pathogens cause morbidity and mortality among consumers of raw and lightly cooked molluscan shellfish. To reduce illnesses, techniques are needed for the extraction and analysis of HAV, NLVs and virulent forms of *V. vulnificus* from edible shellfish tissues. Virus extraction and assay must be made more efficient (time and labor) if it is to be used in practical screening applications. Molecular techniques may be inappropriate for screening processed foods and shellfish which may contain viruses inactivated by chemical, physical, and biological stressors in the environment. Cell culture methods can address the issue of virus infectivity, but no procedures are available for the cell culture of NLVs and the culture of wild-type HAV can be very slow and arduous. New, innovative procedures to propagate and assay these viruses are needed. The objectives of this research are to: (a) develop rapid nucleic acid extraction techniques for molecular detection of HAV and NLVs in molluscan shellfish by RT-PCR-based analyses, (b) develop cell culture-based assays for the enumeration of HAV and NLVs, and (c) evaluate the uptake, distribution and elimination of enteric viruses and vibrios from shellfish maintained under simulated natural conditions with the goal of developing improved shellfish depuration and other purification techniques.

Summary Accomplishments During Entire Project:
This CRIS project is a replacement for 1935-42000-030 project which was scheduled to terminate on 8/31/99. During the short life of this project we identified new chemiluminescent assays for the detection of cytopathic and non-cytopathic hepatitis A viruses and rotaviruses. We made substantial progress in developing unique procedures for the extraction of hepatitis A and Norwalk virus from oyster and clam tissues.

Summary 2000 Accomplishments:
Two new immunological methods, the luminescent immunofocus assay (LIFA) and the luminescent immunofocus inhibition assay (LIF-IA), were developed for the quantitation of cytopathic and non-cytopathic viruses propagated on cell culture monolayers. These methods use enhanced chemiluminescent detection to identify foci (luminescent immunofoci, LIF) of virus-infected cells. Viruses are propagated in susceptible cells under an agarose overlay, inactivated with ultraviolet irradiation, lifted onto nitrocellulose membranes, and probed with virus-specific monoclonal or polyclonal antibody followed by a second antibody conjugated to horseradish peroxidase. Membranes are then treated with a luminol-based detection reagent and exposed to light sensitive film for up to10 min. The film is developed and foci appear as dark, discrete spots which are proportional to the dose of each virus. The LIFA detected both cytopathic and non-cytopathic HAV and simian rotavirus (RV). For the cytopathic HAV, the LIFA and plaque counts were comparable. The LIF-IA was developed for HAV using virus-specific antiserum which effectively attenuated LIF formation. The LIFA and LIF-IA may be completed five days faster than conventional radioimmunofocus assays for HAV and RV and do not require the use of radiolabeled antibodies, offering safety advantages and making these techniques more adaptable for general use. Luminescent immunofocus assays should be useful for the detection and quantitation of virtually any cytopathic or non-cytopathic viruses that can be propagated in monolayer cultures when virus-specific antiserum is available.

As part of an effort to develop a broadly-applicable test for NLVs and HAV in shellfish, a rapid extraction method, suitable for use with one-step RT-PCR-based detection methods, has been developed. The method involves virus extraction using a pH 9.5 glycine buffer, polyethylene glycol (PEG) precipitation, Tri-reagent™ and purification of viral polyA RNA using magnetic polydT beads. This glycine, PEG, Tri-reagent, Poly dT (GPTT) extraction method can be performed in less than 8 h on hard shell clams (*Mercenaria mercenaria*) and Eastern oysters (*Crassostrea virginica*) and, when coupled with RT-PCR-based detection, can yield results in 24 h. Observed sensitivities for spiked shellfish extracts are as low as 0.015 pfu for HAV and 22.4 RT-PCR_{50} units for Norwalk virus. Detection of HAV in live oysters experimentally exposed to contaminated seawater is also demonstrated. An adaptation of this method was used to identify HAV in imported clams that were implicated in an outbreak of viral food-borne illness.

Projected Research Accomplishments During Next 3 Years:
The proposed future research will involve further evaluation of chemiluminescence techniques to detect HAV and NLVs. We will complete the development of rapid virus extraction and RT-PCR assay procedures for shellfish to permit rapid detection of HAV and NLVs. We will also complete an evaluation of certain chemicals reported to enhance virus proliferation (Ca^{+2} and Mg^{+2}, 5-iodo-2'-deoxyuridine-5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole, prostaglandin E1, anti-HAV negative sera, and polyethyleneimine) on HAV plaquability, replication rate, and LIFA focus formation . *Vibrio vulnificus* research will focus on determining the kinetics of *Vibrio vulnificus* uptake and depuration as a function of virulence. We will conclude studies involving in situ immunohistochemistry and hybridization studies to determine the fate of viruses and of virulent and

avirulent forms of *V. vulnificus* within shellfish tissues during laboratory uptake and depuration experiments and recommend improved processing/depuration strategies.

Technology Transfer:
New methods developed for the detection of HAV and NLVs have been transferred to interested parties through peer-reviewed publications, written reports, presentations at scientific meetings and communications with scientists and administrators from State and Federal regulatory agencies. We demonstrated the use of our virus extraction technique for shellfish by detecting HAV in clams that were provided by the US Food and Drug Administration and were implicated in an outbreak of enteric virus illness in New York State. We have had discussions with HAV researchers at the Centers for Disease Control and Prevention about our findings and methods. We have provided researchers at Howard University with Norwalk virus and techniques to try to propagate the viruses. In addition, we are working collaboratively with the University of Delaware on the possible inactivation of HAV using high hydrostatic pressure treatment and our newly developed chemiluminescence assay for HAV. The Lead Scientist is also participating with the U.S. FDA, the CDC, and other State and Federal Scientists, as a member of an expert panel to develop a National surveillance program for food-borne viral illnesses, known as VirusNet. Analytical methods for the detection of human enteric viruses in foods are being evaluated for possible implementation in VirusNet screening.

PUBLICATIONS:

Richards, G.P. Enteric virus contamination of foods through industrial practices: a primer on intervention strategies. Journal of Industrial Microbiology and Biotechnology. (In Press).

Kingsley, D.H., Richards, G.P. Caliciviruses. In: *International Handbook of Foodborne Pathogens*, J. Bier and M.D. Miliotis (Eds.), Marcel Dekker, Inc., New York, NY. (Accepted).

Richards, G.P.,Watson, M.A. Immunochemiluminescent focus assays for the quantitation of hepatitis A virus and rotavirus in cell cultures. Journal of Virological Methods (Submitted).

Kingsley, D.H., Meade, G.K., Watson, M.A., Richards, G.P. A rapid, efficient, extraction method for RT-PCR detection of hepatitis A and Norwalk-like viruses in shellfish (Submitted).

PROCEEDINGS/ABSTRACTS:

Richards, G.P. Enteric virus contamination of shellfish: intervention strategies. Proceedings, 3rd International Conference on Molluscan Shellfish Safety, Long Island, NY, June 19-23, 2000. Abstract, p. 65.

Richards, G.P. Molecular techniques for viruses and their limitations: new frontiers in non-molecular methods. International Association for Food Protection, Atlanta, GA, August 6-9, 2000, Abstract Presentation No. S04, p. 109.

Kingsley, D.H., Meade, G.K., Watson, M.A., Richards, G.P. Pathogenic and human viruses and shellfish: The USDA Seafood Safety Laboratory, East Coast Live 2000 Aquaculture Conference, Annapolis, MD, November 1-4, 2000, Abstract.

Richards, G.P. Development of a quantitative method for hepatitis A virus detection. Proceedings, Joint Meeting of the 52nd Interstate Seafood Seminar and the 45th Atlantic Fisheries Technology Conference. Ocean City, MD, November 28 - December 1, 2000. Abstract p. 26.

5.14

CRIS Title: Identification and Application of Novel Technologies for Detection of Pathogenic Microorganisms in Foods and Environmental Samples
CRIS: 5325-42000-027
Scientists: Stanker LH, Brandon DL, Gaffield W, Wong R, Binder RG, Haddon W
Location: Food Safety and Health Research Unit, WWRC, Albany, CA
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Summary Project Aims:
Methods that will allow for rapid monitoring of pathogens are necessary to guarantee that proper procedures are being followed at all levels of food production, including transportation, processing and preparation. Future strategies for pathogen reduction must be based, in part, on faster, more specific, quantitative measurements of foodborne bacteria as well as a better understanding of how pathogenic organisms are carried through food production and processing streams. Biosensor technology, including antibody-based immunosensing and/or antibody-facilitated sample preparation, can address some of these needs.

The major aim of this CRIS project is to investigate and develop novel technologies for detection, identification, and quantification of foodborne pathogenic microorganisms. This is a new research effort that has only recently been initiated. In order for such methods to have the widest application in the food industry, they need to be highly sensitive methods that are simple to execute, and they must be rapid, cost effective, and be easily interpreted. Problems addressed include: 1) identification of novel sampling methods for capturing, concentrating and detecting bacterial and viral contaminants in foods and environmental samples such as air, water, and soils; 2) development of both chemical- and biochemical-based biosensors; 3) evaluation of newly emerging biosensor and detection technologies for foodborne pathogens; and 4) development of novel reagents suitable for rapid detection and for biosensor applications that are aimed at individual and/or defined groups of microorganisms.

Summary Accomplishments During Entire Project:
This is a new CRIS project. Thus, the accomplishments described below under the 2000 accomplishments represent the initial and cumulative accomplishments of this CRIS.

Summary 2000 Accomplishments:
This is a new program and the research agenda is currently being prepared for peer review. In the past year however a number of monoclonal antibodies that bind specific bacterial pathogens have been further characterized and their application as detection reagents for bacterial pathogens was investigated. Specifically, the binding sites of a set of monoclonal antibodies that bind Campylobacter are being studied. Preliminary studies suggest that these antibodies bind to a bacterial component associated with the external surface of the bacteria and may be useful as reagents to capture and concentrate bacteria from solutions. It is anticipated that these antibodies will be incorporated into assay schemes utilizing immunomagnetic beads as a trapping and concentration step couples with detection by either immunochemiluminesence and/or by time-resolved fluorescence.

The mass spectrometer is a potentially valuable biosensor for bacterial pathogens because it measures bacterial cell components, particularly proteins, with femptomole sensitivity and excellent specificity. We are developing a general proteomics-based approach to detecting bacterial pathogens following our previous success using matrix-assisted laser desorption-ionization (MALDI) mass spectrometry to differentiate Campylobacter at the species level. Similar work is ongoing with E. coli strains. Identifications are based on the measurement of specific biomarker ions in whole-cell preparations of the bacteria. An in house computer program has been developed to correlate data from multiple sample analyses and assist in strain identification..

Research also was completed as part of a previous but related CRIS. Anti-Salmonella monoclonal antibodies were produced and used to evaluate a commercially available biosensor system for detection of this pathogen. As few as 120 Salmonella typhimurium cells were detected.

Projected Research Accomplishments During Next 3 Years:
Initially production of new reagents customized for concentration and identification of pathogenic bacteria will be completed. Initial efforts will utilize the extensive in-house skill in the production of monoclonal and recombinant antibodies. New antibody molecules to a variety of pathogens will be produced using a number of different approaches. New antibody reagents are needed since most monoclonal antibodies for pathogens described in the literature were not produced specifically for use in a biosensor or other rapid instrumental method. Our strategy for each set of antibodies produced to a specific pathogen will be similar. The antibodies will be evaluated for their specificity and sensitivity and for their performance in three separate immunoassay (biosensor) formats: (1) in a standard enzyme-linked immunoassays, (2) using an immunomagnetic chemiluminescent biosensor, and (3) using an ultrasensitive time-resolved fluorescent immunosensor. In addition the binding site of each antibody will be determined in order to bettter understand the binding properties of the antibody. Antibody production should be completed by the end of year 1 and characterization by the end of year two. In each case year three will be devoted to application of the reagent to a targeted commodity or environmental sample matirx. Other molecules known to bind bacteria, i.e., receptors lining the gastrointestinal tract will be isolated and evaluated as substitutes for antibodies. Technology transfer will actively be pursued over the entire 3-year period.

Sample preparation is an integral component of any scheme to detect bacteria and development of rapid extraction methods compatible with rapid bacterial detection methods is critical. Initially using our existing antibodies new methods to separate pathogenic bacteria from complex biological samples will be studies. Following separation of the pathogens from the, in most cases, larger number of non-pathogenic bacteria methods will be developed to concentrate the pathogens in order to perform an identification with no or only limited culturing of the organisms. Bacteria in food products and in environmental samples as well as in manures exist in complex communities (biofilms). We must be able to disrupt these communities, and concentrate the specific pathogens of interest before measurements in real-time will be possible. In the first 2 years we will concentrate on recovery of *E. coli* O157:H7, *Salmonella,* and *Campylobacter* ,from produce and manure samples. In subsequent years we will expand these efforts to include soils, water and if appropriate meats and poultry.

The mass spectrometry studies being conducted represent a novel and highly accurate method to identify pathogenic bacteria. This approach will be pursued over the next three years with the aim of better defining the data bases necessary to identify bacterial strains of *E. col*, and other appropriate pathogens. In addition we will continue our efforts to develop sophisticated, but user-friendly, software to analyze the mass spectral patterns and make a statistically sound assignment of a name. We will: investigate the application of these methods to detect multiple bacteria in a single sample; evaluate a variety of available biosensor systems for their application to pathogen detection in foods and agricultural samples; and evaluate each method as a stand-alone method as well as multiplexing a number of our regents into a system that can either detect different pathogens in a single sample or can detect different properties of a single patghogen but in a single sample. An example of this would be a sensor that detected *E. coli* bacteria, secondly that identified it *as E. coli* O157:H7, and a sensor that detected the presence of the Shiga toxins which are the agents responsible for the hemolytic uremic syndrome often associated I O157:H7 infection.

Technology Transfer:
Technology transfer is an active part of the current CRIS project. Information and technology will be transferred to industry, government regulators, and to the scientific community via publications, collaborations, CRADA's, and Trust Agreements with industry. A CRADA presently is being developed to apply immunomagnetic electrochemiluminescence immunosensors to detection of Campylobacter. In addition, an active collaboration is underway with the manufacturer of a Time-Delayed Fluorescence Immunosensor. This collaboration involves scientist from this CRIS, the CDC, and from USAMRID, aimed at application of this technology to detection of pathogenic bacteria. Specific activities include evaluation of these technologies for pathogen detection in poultry and on fresh produce. A collaboration with a scientist at UC-Davis to develop novel biosensors (based on internal lasing of droplets) aimed at pathogen detection continues. Finally a CRADA to commercalize antibodies to mycotoxins has been completed and antibodies transferred to the industry partner.

PUBLICATIONS:

Dill, K., Stanker, L.H., and Young, C.R. Detection of Salmonella in poultry using a silicon chip-based biosensor. Biochem Biophysical Methods 41:61-67, 1999.

PROCEEDINGS\ABSTRACTS

Harden WF., Lieberman A., Mandrell, R., Haddon, WF. A spreadsheet approach to bacterial identification based on MALDI-TOF spectra of whole cells, Proceedings, 48th Conference on Mass Spectrometry and Allied Topics, Long Beach, CA. June 12-16, 2000 (In Press).

Haddon WF., Harden, HA., Schatzki TF. "2-D Mass Spectral Analysis of Aflatoxin B1 in Peanuts Using Positive APCI," Proceedings, 48th Conference on Mass Spectrometry and Allied Topics, Long Beach, CA. June 12-16, 2000 (In Press).

5.18

CRIS Title: Molecular Systematics and Diagnostics for Parasites of Food and food Animals.
CRIS: 1265-42000-002
Scientists: Rosenthal BM, Hoberg EP.
Location: Biosystematics Unit, PBESL, ANRI, BARC (formerly BNPCU), Beltsville, MD
Contact: 301-504-5408 (P), 301-504-8979 (F); brosenth@anri.barc.usda.gov

Summary Project Aims:
Protozoan parasites, particularly those in the Apicomplexa, cause significant disease in humans, domestic stock and companion animals (e.g., the enteric *Cyclospora*, *Cryptospordium*, and the tissue dwelling *Toxoplasma*, *Neospora*, and *Sarcocystis*), resulting in great economic loss. Either by direct infection of humans or as zoonotic parasites from domestic or sylvatic sources, these parasites represent a threat as foodborne or environmentally transmitted pathogens. Current control measures are inadequate, hampered in part by the extreme difficulty in identifying and diagnosing these microparasites. Whereas certain parasite groups can be identified morphologically by experts, useful criteria for defining species, strains and populations are currently lacking for these protozoans. For some genera such as *Cryptosporidium,* multiple species may parasitize the same hosts, further complicating the determination of parasite diversity. The primary mission of this CRIS is to: (1) Develop accurate and sensitive detection methods based on molecular diagnostic markers for foodborne parasites associated with animal and plant-based foods and from environmental contamination including water; (2) Improve the classification of these organisms according to their evolutionary relationships; (3) Use phylogenetic information to better predict the distribution of parasites among domesticated and wild host species and to predict the risk of introductions into food animals; (4) Refine the definition of parasite species, strains, and populations using molecular phylogenetic approaches. Apicomplexan protozoans including *Toxoplasma*, *Cryptosporidium*, *Neospora, Sarcocystis* and *Cyclospora* and other protozoan and metazoan pathogens constitute the principal focus for research priorities.

Summary Accomplishments During Entire Project:
As a new research program, the activities described for this past year (below) constitute the entire project accomplishments.

Summary 2000 Accomplishments:
The protozoan *Entamoeba histolytica* is a significant waterborne and foodborne pathogen in humans, and the agent of significant disease worldwide. In 2000, the most important accomplishment by BNPCU scientists, in collaboration with a team from the Harvard School of Public Health, was the discovery and characterization of the origins of important biochemical pathways in this protozoan. Investigations established that several fermentation enzymes were acquired from anerobic prokaryotes during the evolution of *Entamoeba*. New information on biochemistry of *Entameoba* fundamentally changes our understanding of parasite behavior in vertebrate hosts and may offer a mechanism on which to base development of effective intervention and control of debilitating infections in humans.

Additional accomplishments in 2000 are related to studies of enteric and tissue dwelling coccidian parasites: (1.) Enteric coccidian parasites are significant pathogens in a diversity of mammalian species, including both domesticated and wild hosts, and identification of species of these microparasites remains challenging. BNPCU scientists in collaboration with those at PBEL refined approaches to identification of coccidians through detailed comparative morphological studies of *Eimeria procyonis* in racoons. Results represent a contribution to knowledge of the diverse genus *Eimeria* and a broader understanding of disease processes associated with enteric coccidia in mammalian hosts. (2.) Coccidian parasites belonging to the genus *Sarcocystis* include species that reproduce sexually in the intestine of opossums, but form tissue cysts in the musculature or central nervous system of their intermediate hosts; significant disease is caused by some species, including for example EPM, or, protozoal myeloencephalitis in horses. BNPCU scientists in collaboration with those in the PBEL identified previously unknown diversity among those organisms and distinguished a recently recognized isolate from *S. falcatula* and *S. neurona*, the causative agent of EPM, showing that both pathogenic and nonpathogenic species may be spread by opossums. Because control of these parasites requires a firm understanding of parasite identity, relationships, host association and geographic distribution, these molecular studies contribute directly to defining patterns of disease for *Sarcocystis*. (3.) Clinical reports and immunological surveys indicated that Sarcocystis neurona, known previously only in the United States, might be a prevalent pathogen in horses of South America. BNCPU scientists working with colleagues in the PBEL and an international team of collaborators, demonstrated that species of opossums endemic to South America are infected by *S. neurona* parasites. Because this recently recognized parasite is now known over a broad geographic range and in an increasingly diverse array of opossum species, this work demonstrates a far greater potential impact for this pathogenic species than was previously imagined. (4.) Little is known about the natural prevalence of infection of *Sarcocystis* parasites in opossums in the vicinity of American farms, despite the fact that one of these species, *Sarcocystis neurona*, is a major cause of neurological disease in horses. In order to define the exposure of farm animals to pathogenic and non-pathogenic parasite species, BNCPU, PBEL and collaborating scientists conducted a prospective survey of parasite prevalence in opossums inhabiting the vicinity of horse farms in Mississippi. Over half of the opossums examined were shown to be infected with *Sarcocystis neurona*, showing their great potential to contaminate pastures with this neuropathogenic parasite.

Projected Research Accomplishments- Next 3 Years:

Molecular systematics techniques and methods are at the center of our ability to identify parasites, develop rapid and accurate diagnostic procedures and thus the foundation for understanding the basis for host associations, geographic distribution and patterns of disease. Multifaceted research within the context of food safety issues and animal health will focus on the application of molecular techniques to studying genetic variation in enteric and tissue-cyst forming parasites of economic and potential economic significance. (1.) A collaborative study with researchers at the Tufts University School of Veterinary Medicine of genetic variation among human and bovine isolates of the pathogen *Cryptosporidium parvum* will culminate during 2001. Using a unique collection of cloned parasite lineages, variation at each of several genetic loci will be characterized in order to

determine how these organisms diversify and whether animal and human parasites exchange genes. The work will be significant in defining the extent to which such parasites, the causative agents of major diarrheal disease in humans and livestock, are capable of adapting to new hosts during the course of an infection. (2.) Vaccines offer one important means of protecting against coccidiosis in poultry, however considerable variation has been observed among isolates of *Eimeria maxima* parasites. A project to determine the genetic relationships among immunologically distinct isolates of *Eimeria maxima*, to be completed in 2001, will support the rational design of vaccines that protect against the widest possible range of economically damaging parasites. (3.) Obtaining quality DNA from formalin fixed tissues remains a technical challenge, but if resolved historical studies of parasite genetic diversity both among species and populations will be possible for the first time. By 2002, we expect to enhance our sampling of genetic variation among animal parasites by refining methods to obtain DNA from formalin-fixed specimens. An initial anticipated outcome of this method will be a wide-ranging comparative study of *Sarcocystis* spp. from diverse domesticated and wild animal hosts as a basis for species-identification and an examination of the evolutionary relationships for this group of pathogenic parasites. (4.) Ongoing projects in coccidian systematics will be augmented by studies of selected parasitic nematodes in ruminants through 2003. Interdisciplinary research will help define the extent to which climate change is affecting the exposure of food animals to parasites historically restricted to wild ruminants; this focus deals with invasive and emerging pathogens and the interface of agricultural and natural ecosystems.

Technology Transfer:
Results from these studies are being transferred to action agencies and stakeholders though collaborations, peer-reviewed publications; presentations at scientific and management meetings, and other public presentations. Collaborations with academic scientists include: A memorandum of understanding with faculty of the Tufts University School of Veterinary Medicine for collaborative research on genetic diversity of protozoans.(2) Invited lectures: a) University of Maryland; b) Smithsonian Institution; c) Helminthological Society of Washington.

PUBLICATIONS:
Submissions to peer-reviewed journals include 8 manuscripts based on research under this program.

PROCEEDINGS/ABSTRACTS
Rosenthal, BM, Rozas, J., and Spielman, A. Diversifying Selection on immunogenic proteins of lyme disease spirochetes. Abstracts, Annual Meeting, American Society for Tropical Medicine and Hygiene November 1999. American Journal of Tropical Medicine and Hygiene 1999. v. 61(3) p.430

Rosenthal, B, Rozas, J., and Spielman, A. Selective constraints on genes in the agent of Lyme Disease. Abstracts, Annual Meeting Society Molecular Biology and Evolution. 2000. p.223.

CRIS Title: Molecular Genomics of Plant Pathogens and Food Safety Microorganisms
CRIS: 3620-42000-022
Scientists: Kurtzman CP, Labeda DL, Nakamura, LK, Peterson SW, O'Donnell K, (vacant)
Location: Microbial Properties Research Unit, NCAUR, Peoria, IL
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Summary Project Aims:
Basic and applied genomics research to understand population genetics and species boundaries of: plant pathogenic and mycotoxigenic species of *Fusarium, Aspergillus,* and *Penicillium;* biocontrol species of *Bacillus*; plant pathogenic species of *Streptomyces;* and biocontrol and food spoilage yeasts. This information is essential for understanding occurrence, transmission and control of pathogens and toxin producing microorganisms.

Summary Accomplishments During Entire Project:
The Research Unit has developed species-specific multi-gene sequence databases for all known species of *Fusarium, Aspergillus, Penicillium* and ascomycetous yeasts, and is initiating similar work on *Streptomyces* and *Bacillus*. These databases allow, for the first time, accurate identification of species in these groups, and this information is essential to understanding genetic diversity in the wheat scab pathogen *Fusarium graminearum*, aflatoxin producing species of *Aspergillus*, and biocontrol and food spoilage yeasts. The data provide a means for detection of the species in the environment, rapid laboratory identification and understanding biogeographical distribution and consequent risk assessment. A practical outcome of this work is development of rapid detection kits for pathogens and food spoilage species, which is being undertaken through two CRADAs.

Summary 2000 Accomplishments:
We have conducted genomic studies of the eight lineages comprising *Fusarium graminearum*, detected new aflatoxigenic species of *Aspergillus*, discovered new biocontrol and food spoilage yeasts, detected new actinomycetes pathogenic to horses and demonstrated that the mosquito biocontrol pathogen is not *Bacillus sphaericus*, but a species new to science.

Projected Research Accomplishments During Next 3 years:
Direction of this project is being expanded to include genomic studies of the foodborne bacterial pathogens *Listeria monocytogenes* (Position, filled December) and *Bacillus cereus*. These studies will focus on unique gene sequences common to pathogenic strains that will explain pathogenicity and allow rapid detection of the pathogens in the environment and in food products. Work on the pathogenic and mycotoxigenic molds *Fusarium*, *Aspergillus* and *Penicillium* will include expanded genomics studies to understand environmental distribution and to provide rapid means for detection. Work on food and beverage spoilage yeasts will focus on population structure and distribution of spoilage species and molecular methods for their detection. Databases will be expanded for more accurate identification of biocontrol species.

PUBLICATIONS:

O'Donnell, K., Kistler, H.C., Tacke, B.K., Casper, H.C. Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. Proceedings of the National Academy of Sciences USA. 2000. v.97(14). p.7905-7910.

Labeda, D.P., Kroppenstedt, R.M. Phylogenetic analysis of *Saccharothrix* and related taxa: proposal for *Actinosynnemataceae* fam. nov. International Journal of Systematic and Evolutionary Microbiology. 2000. v.50. p.331-336.

Kurtzman, C.P. Four new yeasts in the *Pichia anomala* clade. International Journal of Systematic and Evolutionary Microbiology. 2000. v.50. p.395-404.

Palys, T., Berger, E., Mitrica, I., Nakamura, L.K., Cohan, F.M. Protein-coding genes as molecular markers for ecologically distinct populations: the case of two *Bacillus* species. International Journal of Systematic and Evolutionary Microbiology. 2000. v.59. p.1021-1028.

Kurtzman, C.P. Three new ascomycetous yeasts from insect-associated arboreal habitats. Canadian Journal of Microbiology. 2000. v.46. p.50-58.

Aoki, T., O'Donnell, K. Morphological characterization of *Gibberella coronicola* sp. nov., obtained through mating experiments of *Fusarium pseudograminearum*. Mycoscience. 1999. v.40. p.443-452.

Ito, Y., Peterson, S.W., Goto, T. Properties of *Aspergillus tamarii*, *A. caelatus* and related species from acidic tea field soils in Japan. Mycopathologia. 1999. v.144. p.169-175.

Fonseca, A., Fell, J.W., Kurtzman, C.P., Spencer-Martins, I. *Candida tartarivorans* sp. nov., an anamorphic ascomycetous yeast with the capacity to degrade L(+)- and meso-tartaric acid. International Journal of Systematic and Evolutionary Microbiology. 2000. v.50. p.389-394.

Kurtzman, C.P. Systematics and taxonomy of yeasts. Ernst, J.F., Schimidt A., editors. Karger. Freiburg, Germany. Dimorphism in Human Pathogenic and Apathogenic Yeasts. 2000. p.1-14

O'Donnell, K. Molecular phylogeny of the *Nectria haematococca-Fusarium solani* species complex. Mycologia. 2000. v.92(5). p.919-938.

Benny, G.L., O'Donnell, K. *Amoebidium parasiticum* is a protozoan, not a Trichomycete. Mycologia. 2000. v.92(6). p.1133-1137.

O'Donnell, K. Nirenberg, H.I., Aoki, T., Cigelnik, E. A multigene phylogeny of the *Gibberella fujikuroi* species complex: detection of additional phylogenetically distinct species. Mycoscience. 2000. v.41. p.61-78.

PROCEEDINGS/ABSTRACTS:

Nakamura, L.K., Shida, O., Takagi, H., Komagata, K. *Bacillus pycnoticus* sp. nov. and *Bacillus neideii* sp. nov., two new round-spored organisms. 100th General Meeting of the American Society for Microbiology. May 2000. Abstract p.629.

Labeda, D.P. *Crossiella* gen. nov., a new genus related to *Streptoalloteichus*. 100th General Meeting of the American Society for Microbiology. May 2000. Abstract p.629.

Peterson, S.W. Phylogenetic placement of the genera *Sagenoma* and *Dichotomomyces* in the Trichocomaceae. Annual Meeting of the Mycological Society of America. July 2000. Abstract p.41.

O'Donnell, K. Comparative genomics of *Fusarium*: advances from germplasm conservation. American Phyotopathogical Society. Aug 2000. Abstract p.S96.

CRIS Title: Control of Pathogenic and Spoilage Bacteria on Red Meat
CRIS: 5438-32000-014
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Location: Meats Research Unit, U.S. MARC, Clay Center, NE
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Summary Project Objectives:
Develop antimicrobial intervention strategies for slaughter, processing, and fabrication, and determine intervention effects on microbial safety, spoilage, and ecology of the resulting products (e.g., carcass, subprimal, trim, ground beef). Develop representative sampling procedures and sensitive, accurate, and rapid diagnostic methods to detect foodborne pathogens and other microbial contaminants. Determine the effects of previous environment and/or intervention procedures on the induction of adaptive responses in bacteria that result in increased antimicrobial resistance and/or virulence. Determine and characterize molecular mechanisms of pathogen resistance and virulence development, and develop procedures to prevent these occurrences.

Summary Accomplishments During Entire Project:
Accomplishments over the life of this project include numerous studies regarding carcass antimicrobial spray wash treatments, including high pressure water and hot water and such compounds as organic acids (mainly lactic and acetic acids), plant-derived chemicals (saponin, herb extracts), and synthetic chemicals (cetylpyridinium chloride, chlorine compounds, trisodium phosphate, hydrogen peroxide, and triclosan), and their effectiveness in reducing viable bacteria on carcass tissues. Bacterial populations examined have included both spoilage and pathogenic bacteria such as *Escherichia coli* O157:H7, *Salmonella* spp., and *Listeria* spp., as well as newly recognized pathogens (e.g., *Salmonella* DT104), and phenotypic variants of enterohemorrhagic *E. coli* (rough and smooth variants of *E. coli* O157, and *E. coli* O26 and O111) In general, these spray washes affected reductions of 90 to 99.9% of bacteria numbers per sq. cm. of carcass tissue. Carcass steam vacuum machines were demonstrated to greatly reduce microbial loads on carcasses (>99.9% bacteria per sq. cm.). Water, hot water, and antimicrobial spray washing interventions are now widely used by the red meat industry. Steam vacuums are in standard use in most medium and large meat slaughter operations in the U.S.

Nisin, an antimicrobial protein produced by bacteria, was incorporated into synthetic meat packaging film and shown to reduce growth of spoilage bacteria on refrigerated meat. Nisin also was used with a commercially available meat binding system (Fibrimex™) to reduce bacteria on fresh or vacuum-packaged meat surfaces and was effective as a way of reducing undesirable bacteria in restructured meat products made from whole muscles or trim that incorporate surface tissues into product interior. A multiple hurdle antimicrobial intervention process for beef trim was developed and validated for reducing microbial contamination in the resulting ground beef. Rapid carcass microbial load tests were developed that are practical for use in on-line HACCP process monitoring and verification. A rapid microbial ATP test was developed and validated that indicates the carcass microbial load in less than 5 minutes with a

good correlation with standard plate count methods. This method is a good predictor of the presence/absence of *E. coli* biotype 1, which is the FSIS fecal contamination indicator organism of choice for red meat carcasses. This test is commercially available and has users worldwide for testing meats and further processed products. A more representative and rapid method for sampling meat trim was developed that uses the meat juices or purge from 2,000-lb. standard beef trim combos to assess the microbial content. Numerous carcass sampling method evaluations were conducted on request from the FSIS, including evaluations of sponge and excision sampling, and some of this data was included in the FSIS Pathogen Reduction/HACCP regulations. Bioluminescent *E. coli* O157 was macro-imaged directly from the surface of inoculated beef tissues, allowing totally undisturbed or non-destructive sampling of and observation of bacteria. This real-time assessment of microbial attachment to carcass surfaces is expected to facilitate evaluation of carcass decontamination procedures and mechanistic studies of microbial contamination of beef carcass tissues.

Summary 2000 Accomplishments:
Currently, antimicrobial intervention processes used in the slaughter industry are focused on decontamination of the carcass; however, in the normal process of breaking down the carcass into smaller meat cuts and trim, there are additional opportunities to not only spread any remaining bacterial contamination but to add contamination from equipment and tools, and by handling by multiple personnel throughout the process. Combination treatment processes for the microbial decontamination of pork trim were designed and evaluated, for use at the last possible step prior to grinding. These treatment processes were shown to reduce and control populations of fecal bacteria on pork trim and in the resultant ground pork. This work provides the meat industry with a process to decontaminate meat trim, which can improve both the microbial safety and shelf life of ground pork products and may assist processors in meeting the proposed *Salmonella* performance standards for fresh pork sausage. Pathogenic bacteria may be injured rather than killed when subjected to processes designed to inactivate or inhibit them in foods (e.g. heat, freezing, or low pH), and this injury may prevent their growth and subsequent detection on the selective media used to detect their presence in the food.

Novel methods to recover and enumerate sublethally injured bacteria were developed and validated. An agar underlay method was demonstrated to recover heat-, acid-, and freeze-injured pathogens, including *E. coli* O157:H7, from buffer and meat matrices, while still allowing adequate selectivity and differentiation for isolation, detection, and enumeration. A two-fold dilution method was demonstrated to recover and allow the selective enumeration of heat-injured coliforms from beef carcass swab samples. Use of these methods will improve the accuracy of detection and enumeration of injured yet viable pathogens in meats and other foods by the industry. Rapid, sensitive microbial tests are needed for near-immediate detection of fecal and microbial contamination that may occur during the meat production process. Methods to rapidly detect microbial contamination on beef carcasses were developed and validated. A commercially available color test that detects molecules on the surface of fecal bacteria (*Limulus* amoebocyte lysate assay) was validated to gauge levels of beef carcass microbial contamination within 10 minutes. A commercially available fluorescence-based microbial phosphatase test kit was validated to estimate beef carcass microbial contamination within 10 minutes. Use of these tests would allow the industry to respond more quickly to

correct problems that result in contamination of carcasses. Highly sensitive and rapid procedures are needed for detection of *E. coli* O157:H7, which has been declared an adulterant in ground beef and other non-intact raw beef products. The BAX system, which is a commercially available test based on polymerase chain reaction detection, was evaluated for screening bovine carcass sponge samples for the presence of *E. coli* O157:H7. The BAX system was demonstrated to be a rapid, reliable, and simple method to screen carcass sponge samples for *E. coli* O157:H7 and showed excellent agreement with a demonstrated culture method. The use of this method will allow processors to rapidly detect this organism in bovine carcass samples. Research examining the growth and survival of *E. coli* O157:H7 during the refrigerated and frozen storage of ground beef have primarily utilized laboratory strains of this bacterium, and this may not adequately reflect natural ground beef contamination with this organism. The growth and survival of recent natural bovine isolates of *E. coli* O157:H7 during refrigerated and frozen storage of ground beef were determined. Recent bovine isolates of *E. coli* O157:H7 in ground beef were demonstrated to exhibit growth and survival characteristics similar to those that have been reported for laboratory strains. These results confirm previous reports that storage temperature is very important to limit growth of *E. coli* O157:H7 and that storage at 7°C permits growth of this organism in ground beef.

Projected Research Accomplishments During Next Three Years:
Additional processes and antimicrobials will be developed and examined for use in microbial decontamination of beef and pork trim, and the effects on microbial and functional quality of the resulting ground products will be determined. Beef trim interventions will be developed with the goal of reducing gas-forming bacteria that cause ground beef chub blow-ups. Recommendations to the industry for appropriate sample handling procedures for recovery of *E. coli* O157:H7 from ground beef will be developed. The acid resistance status of *E. coli* O157:H7 as shed in bovine feces will be determined. The variation of acid resistance and ability to adapt to acidic conditions among recent bovine isolates of enterohemorrhagic *E. coli* will be determined. The technique of prokaryotic differential display has been developed, and will be used to identify genes involved in the development of tolerance to low pH and organic acids by *E. coli* O157:H7. The biochemical and ultrastructural changes of molecular components of the outer membrane of *E.coli* O157:H7 in response to organic acids and low pH will be determined. The expression of *E. coli* O157:H7 genes involved in acid resistance development upon organic acid spray washing of beef will be determined. The expression of toxin and virulence genes of *E. coli* O157:H7 exposed to antimicrobial agents used in interventions will be examined. Procedures to control acid resistance development and outer membrane adaptations by *E. coli* O157:H7 will be developed, utilizing knowledge of the molecular mechanisms of acid adaptation by this organism. Improved intervention processes to eliminate *E. coli* O157:H7 from meat will be developed.

Technology Transfer:
Information on various accomplishments related to antimicrobial interventions to reduce both pathogenic and spoilage bacteria on carcasses and trim has been presented to scientific and industrial groups that include microbiologists, meat industry professionals, livestock breeders and producers, and other professionals that are potential end-users of the science and/or technologies. ARS researchers have been contacted by and consulted with a corporate research

& development scientist, to answer questions, solve problems, and discuss potential approaches for further developments and uses of hydroxyapatite concentration of bacteria for the detection of low numbers of foodborne pathogens from food and water samples.

PUBLICATIONS:

Cutter, C. N. Antimicrobial effect of herb extracts against *Escherichia coli* O157:H7 , *Listeria monocytogenes*, and *Salmonella* Typhimurium associated with beef. Journal of Food Protection. May 2000. v. 63 (5). p. 601-607.

Cutter, C. N., Dorsa, W. J., Handie, A., Rodriguez-Morales, S., Zhou, X., Breen, P. J., and Compadre, C. M. Antimicrobial activity of cetylpyridinium chloride washes against pathogenic bacteria on beef surfaces. Journal of Food Protection. May 2000. v. 63 (5). p. 593-600.

Kang, D. H. and Siragusa, G. R. 1999. Agar underlay method for recovery of sublethally heat-injured bacteria. Applied and Environmental Microbiology. Dec. 1999. v. 65 (12). p. 5334-5337.

Siragusa, G. R. Statistical validation of the Track-Dilution plating methods from ground beef and carcass surface samples. Journal of Rapid Methods and Automation in Microbiology. 1999. v. 7. p. 155-161.

PROCEEDINGS/ABSTRACTS:

Rivera-Betancourt, M. and Cutter, C. N. A comparison study between rough and smooth phenotypes of *Escherichia coli* O157:H7 associated with beef surfaces following spray interventions. Abstracts of the 100th General Meeting of the American Society for Microbiology, Los Angeles, CA. May 2000. Abstr. P-46. p. 522.

Koohmaraie, M. Effective intervention methods for small processors. Presented at the Food Safety Work Conference on Reducing Foodborne Illness: Advancing the Adoption of Technologies, Washington, D.C. December 13-14, 1999.

Siragusa, G. R. Overview of meat safety research and future directions in the new millenium. Presented at the AOAC International Annual Meeting, Houston, TX. September 26-30, 1999.

Cutter, C. N., J. L. Willett, and G. R. Siragusa. Improved activity of nisin-incorporated plastic by formulation change and addition of food grade chelators. Presented at Conference on Bacteriocins: Progress in Food Applications and Regulatory Aspects, Horsholm, Denmark. November 8-9, 1999.

CRIS Title: Development of On-line Verification and Intervention Procedures for HACCP in Slaughter/Processing Systems
CRIS: 5438-42000-002
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Summary Project Aims:
Research projects are designed to support the development of effective HACCP plans by 1) identifying new and important critical control points (CCPs) and 2) discovering unique information regarding the nature of specific pathogens, which in turn will guide development of better methods to specifically detect and reduce or eliminate red meat pathogen contamination. The specific project goals are to: develop practical antimicrobial intervention strategies for slaughter, processing, and fabrication that maintain product quality, develop improved sampling, detection, and identification methods for monitoring and verification of CCPs, determine and characterize molecular mechanisms and sources of pathogen virulence acquisition, and develop procedures to prevent these occurrences, and investigate genomic and virulence characteristics of pathogens and apply this information to the elimination of pathogens from the meat supply.

Summary Accomplishments During Entire Project:
Bacterial cross-contamination of swine carcasses frozen in a continuous liquid nitrogen immersion system was demonstrated, identifying a potential critical control point that could affect both the microbial safety and shelf life of the products. This CCP may be important to both the microbial safety and shelf life of the product, especially if it is thawed and stored under refrigeration for an extended period of time prior to further processing or heating. The use of a lactic acid spray wash before the carcasses entered the freezing system reduced the incidence of cross-contamination. This treatment reduced the levels of bacteria introduced into the system, also suggesting that other strategies to limit the number of bacteria entering the system would limit cross-contamination. Hydroxyapatite was shown to be useful for concentrating low numbers of *Salmonella typhimurium* from ground beef and beef carcass sponge samples in preparation for PCR detection procedures. This method shortens the time required to detect the bacteria by eliminating the need for time-consuming growth enrichments and also provides a means to analyze larger, more representative samples for better recovery of bacteria present in low numbers.

Screening of several strains of *E. coli* O157:H7 revealed that different strains exhibit a range of relative acid resistance and ability to develop tolerance to acid. In addition, it was demonstrated that *E. coli* O157:H7 acid resistance and acid adaptation can affect the efficiency of organic acid spray wash treatments commonly used to decrease bacterial levels on beef carcasses; some *E. coli* O157:H7 strains were found to be more resistant to killing by acid after acid adaptation that may occur in the bovine gut. This study suggests that it is important to confirm that organic acid spray washes work against *E. coli* O157:H7 in its probable natural state. It was demonstrated a strong association between the level of generic microbial contamination and the

incidence of *E. coli* biotype I on beef carcasses. As part of its pathogen reduction plan, the FSIS has required that meat and poultry establishments document verification of process control by testing finished carcasses for the presence of *E. coli* biotype I. Methods to determine the level of generic contamination in near real time do exist; use of these methods would provide the processor with a HACCP monitor to quickly detect and correct process deviations associated with fecal contamination. It has been determined that antimicrobial treatments that substantially lower the total number of bacteria on beef carcasses do not permit the unchecked growth of surviving *E. coli* O157:H7 during proper refrigeration. This information should allay fears that antimicrobial treatments were eliminating "good" bacteria able to compete with *E. coli* O157:H7 and keep it from growing.

Summary 2000 Accomplishments:
The relationship between cattle contamination and subsequent carcass contamination had never been examined in the U.S. In conjunction with ARS preharvest food safety scientists the prevalence of *E. coli* O157:H7/NM in cattle feces and on hides and carcasses was determined at four large U.S. beef processing plants. In this study, the unexpectedly high number of animals per lot that entered the plant carrying *E. coli* O157:H7/NM corresponded to the number of carcasses initially contaminated with the bacteria, but very few carcasses were still contaminated after processing. These results have contributed to food safety and policy debates regarding commonness of *E. coli* O157:H7/NM, usefulness of current sampling procedures, and strategies to eliminate *E. coli* O157:H7/NM contamination of the beef supply. A method was needed to examine *E. coli* O157:H7 responses to the environment they encounter on beef carcasses. Different ways to study changes in the bacteria as they are exposed to various conditions while on beef tissue, such as lower pH due to acid washes, were evaluated. A method was developed to detect gene expression activities in *E. coli* O157:H7 recovered directly from beef carcass tissue, providing a critical tool to explore the adaptation responses of *E. coli* O157:H7 to its environment on beef surfaces.

Projected Research Accomplishments During Next 3 Years:
Establish the contribution of various contamination sources to the transfer of *E. coli* O157:H7 onto beef carcasses. Demonstrate the relatedness of *E. coli* O157:H7 strains that cause disease and those from cattle that have not yet been associated with disease. Improve recovery and detection of *E. coli* O157:H7 from beef carcass sponge samples in order to increase sensitivity. Identify components of beef tissue to which *E. coli* O157:H7 can attach, a possible mechanism that bacteria use to remain on the meat. Uncover specific sources of bacterial carcass contamination and the processing steps involved (CCPs). Determine the potential for *E. coli* O157:H7 to bind to other bacteria that could help in attaching the pathogen to beef carcasses. Discern the ability of *E. coli* O157:H7 to transfer disease-associated genes to and from other bacteria found in its environment. Characterize disease-associated traits of *E. coli* O157:H7 strains that have not yet been associated with disease.

Technology Transfer:
Presentations were made regarding the relationship in prevalence of E. coli O157:H7/NM in cattle and on carcasses at U.S. beef processing plants. Popular press coverage included AP/Reuters National Academy of Sciences, and USDA-ARS News Service press releases as

well as articles in daily newspapers and the National Meat Association newsletter. Presentations were made at the annual USDA-ARS/FSIS Food Safety Research Planning Meeting and a phone interview was conducted with county extension agents in Colorado.

PUBLICATIONS:

Berry, E.D., Cutter, C.N. Effects of acid adaptation of Escherichia coli O157:H7 on efficacy of acetic acid spray washes to decontaminate beef carcass tissue. Applied and Environmental Microbiology. 2000. v. 66 (4). p. 1493-1498.

Elder, R.O., Keen, J.E., Siragusa, G.R., Barkocy-Gallagher, G.A., Koohmaraie, M., Laegreid, W.W. Correlation of enterohemorrhagic Escherichia coli O157 prevalence in feces, hides and carcasses of beef cattle during processing. Proceedings of the National Academy of Sciences USA. 2000. v. 97 (7). p. 2999-3003.

PROCEEDINGS/ABSTRACTS:

Barkocy-Gallagher, G.A., Siragusa, G.R., Keen, J.E., Elder, R.O., Laegreid, W.W., Koohmaraie, M. Prevalence and relatedness of Escherichia coli O157 isolates recovered from beef cattle at processing. Society for Industrial Microbiology. 2000. Abstract p. 26.

Barkocy-Gallagher, G.A. Genomic comparison by pulsed-field gel electrophoresis of Escherichia coli O157:H7 cattle isolates and clinical isolates. American Society for Microbiology. 2000. Abstract p. 521.

6.8

CRIS Title: Quantitative Determination of Pathogen Reduction During Animal Slaughter and Food Processing
CRIS: 1935-42000-034
Scientists: Luchansky JB, vacancy (vice Palumbo S), Medina M, Feder I
Location: Microbial Food Safety Research Unit, ERRC, Wyndmoor, PA
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Summary Project Aims:
In the US, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella*, and *Campylobacter* remain pathogens of current concern. Also in the US, the major vehicles are foods of animal origin. New techniques are needed by the food processing industry to reduce or eliminate these bacteria from foods. Large quantities of meats and meat products have been recalled recently due to detection of pathogens in these foods. The objectives of these studies are to determine steps in swine slaughter and carcass dressing which are capable of reducing or eliminating bacteria from carcass surfaces and to develop new strategies to prevent attachment of and/or to detach pathogens from animal surfaces. These objectives will be accomplished by studying the bacterial levels on swine carcasses after different operations, by studying the mechanisms of bacterial attachment to connective tissues, and by developing model systems to screen for compounds that may inhibit attachment and compounds that may detach adhering bacteria from animal tissues. These compounds may be used as carcass sprays prior to evisceration. This approach will be applied to inhibition and detachment of *Salmonella* and other bacteria from poultry and swine carcasses.

Summary Accomplishments During Entire Project:
A. To evaluate and determine critical control points of trace contamination in the swine slaughter and processing facility, *Aeromonas* was used as an indicator organism. The step-by-step evaluation of the slaughter and dressing operations demonstrated that singeing prior to evisceration is a critical control point of contamination in the swine slaughter and dressing operation. In addition, the swine slaughter plant environment was surveyed for the presence of *Campylobacter* spp. Over 60% of the swine feces tested from swine carcasses on the gambrel table were positive for *Campylobacter* spp.

B. The effect of formulation and application of a biopreservative to control *L. monocytogenes* in a ready-to-eat product was evaluated. Incorporation of potassium lactate (2.8%) as an ingredient and the sample application of *C. piscicola* and its associated bacteriocin resulted in an appreciable per gram reduction of *L. monocytogenes* growth in vacuum packaged hotdogs.

C. A polyanionic polysaccharide, carrageenan, was used to reduce and eliminate bacterial attachment to the connective tissues and fascia within meat. Data from the attachment studies revealed that *E. coli* O157:H7 and *Salmonella* preferentially bound to collagen fibrils of the connective fascia. This binding was inhibited by carrageenan suggesting that the bacterial surface lipopolysaccharides play a role in attachment and, that lipopolysaccharides have a

similar structure to the carrageenans. From these studies, new surface rinse techniques are being proposed for inhibition and detachment of bacteria from meat and poultry surfaces.

Summary 2000 Accomplishments:

A. Processing plant water, plant equipment, and the swine were surveyed for the presence of *Aeromonas* spp. Standard biochemical tests in combination with API 20E test strips were used to identify *Aeromonas*. Riboprinting and pulsed field gel electrophoresis revealed that the live animals introduced new strains of *Aeromonas* spp. into the plant environment each day. These strains were the source of plant environment recontamination and subsequent swine carcass contamination. When the incidence of *Campylobacter* was evaluated, it was demonstrated that *Campylobacter* could be detected at several sites within the carcass handling sequence and at monthly intervals. *Campylobacter* spp. that were isolated from swine carcasses were identified as *C. coli* and *C. jejuni.*

B. Listeria monocytogenes was inoculated onto the surfaces of hot dogs prepared with and without potassium lactate (2.8%). Groups of *L. monocytogenes* spiked hot dogs were also inoculated with a bacteriocin-producing strain of *Carnobacterium piscicola* (10,000 cfu per gram). The hot dogs were vacuum packaged and stored at 4 and 8 deg C. The effectiveness of the presence of a *C.piscicola* bacteriocin on the inhibition of *L. monocytogenes* growth in hot dogs containing lactate was significant when hot dogs were stored under refrigeration (4 deg C) and under an abuse food storage temperature (8 deg C). *L. monocytogenes* was not inhibited on hot dogs that did not contain *C. piscicola* or lactate (4 deg C and 3 deg C), or that contained *C. piscicola* or lactate singly, under an abuse food storage temperature (8 deg C).

C. It was demonstrated, using a BIAcore biosensor, that *Salmonella* and *E. coli* O157:H7 binds to collagen, myosin, laminin, actin, and fibronectin. It was also shown that K-carrageenan is an effective inhibitor in the binding of these pathogenic bacteria to poultry skins and beef carcasses. Detachment of these bacteria was achieved by using arginine followed by a Tween 80/NaCl mixture or by using guanidine-HCl. Stereo fluorescence microscopy studies demonstrated that *E. coli* JM109 GfPur, an *E. coli* expressing a green fluorescent protein, preferentially binds to the connective tissue (fascia) of chicken skin. This binding was also inhibited by K-carrageenan.

Projected Research Accomplishments During Next 3 Years:

A. Identify the critical control points during swine slaughter and carcass dressing and develop appropriate interventions to eliminate pathogens from carcasses. Provide new information on processing techniques to reduce or eliminate pathogens from meat or poultry foods.

B. Evaluate the in vitro inhibition and detachment techniques under actual conditions in a food processing facility. Using the new surface rinse techniques, determine the incidence and levels of *Salmonella*, *E. coli* O157:H7, aerobic plate counts, and coliforms on poultry and meat samples. Investigate and understand the molecular basis of the attachment mechanism and the role of collagen, and bacterial surface lipopolysaccharide and O antigen.

6.10

Technology Transfer:
A. Results of the *Aeromonas* evaluation were discussed directly with our cooperating swine slaughter facility.

B. A confidentiality agreement with Dr. B. Bradley of RMR laboratories for the utilization of surface rinse solutions and the vacuum sampling unit to enhance detachment of pathogens from meat and poultry carcasses has been signed. A confidentiality agreement was signed with Dr. A. R. El-Koubysi of PMI, Inc. for the development of assays for the detection of E. coli O157:H7 using the Spreeta Kit (a SPR biosensor). Exploratory Technology Transfer is being discussed with Ingredient Solutions (Dundee, IL), Air Liquide (Countryside, IL), Dupont (Wilmington, DE), and FMC (Princeton, NJ).

PUBLICATIONS:

Medina, M. B. Method of detaching bacteria from, or of inhibiting bacterial attachment to, animal or poultry carcasses or pieces thereof. 2000. Provisional US Patent Application 0164.98.

Palumbo, S.A., Stelma, G.N., Jr., Abeyta, C. The *Aeromonas hydrophila* Group. Lund, B., Baird-Parker, T., Could, G., editors. Aspen Publishers, Inc., Gaithersburg, MD. In The Microbiological Safety and Quality of Food. 2000. v. II. p. 1011-1028.

Palumbo, S. A., Klein, P., Capra, J., Eblen, S., Miller, A.J. Comparison of excision and swabbing methodologies to determine the microbiological quality of swine carcass surfaces. Food Microbiology.1999. v. 16. p. 459-464.

PROCEEDINGS\ABSTRACTS:

Feder, I. Polymerase chain reaction confirmation of microbes: post harvest concerns. In Handbook for Gala 20th Anniversary Rapid Methods and Automation in Microbiology Workshop. 2000. p. 63-73.

Palumbo, S.A. Use of rapid methods in swine slaughter and dressing operations. In Handbook for Gala 20th Anniversary Rapid Methods and Automation in Microbiology Workshop. 2000. p. 152-180.

Yu, S.-L, Palumbo, S.A. Enumeration of *Aeromonas* for verification of the hygienic adequacy of swine carcass dressing processes. In Handbook for Gala 20th Anniversary Rapid Methods and Automation in Microbiology Workshop. 2000. v. 20. p.43-52.

CRIS Title: Development of Technology for Automated On-Line Inspection of Animal Carcasses
CRIS: 1265-42000-007
Scientists: Chen Y-R , Chao K, Kim MS, Lefcourt A, Delwiche SR
Location: Instrumentation and Sensing Laboratory, BARC, Beltsville, MD
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Summary Project Aims:
The U.S. poultry industry and FSIS need an on-line automated machine inspection system that will accurately screen for wholesome poultry carcasses, therefore allowing inspectors to visually inspect a small number of carcasses rejected by the machine. Such a system, placed strategically in poultry slaughter plants, would help increase throughput of products, minimize problems of human inspection error and variability, improve the effectiveness of the federal inspection program, and ease consumer concerns about the safety of USDA-inspected poultry and poultry products. The first objective is to develop accurate poultry inspection equipment for on-line differentiation of wholesome from unwholesome poultry carcasses. This on-line automated poultry inspection system will be able to operate unattended for long periods of time, and can re-calibrate itself as new populations of chickens are encountered, incorporating the expertise of veterinarians and floor inspectors. It will be low-cost, able to operate with minimum human intervention, and able to maintain its accuracy despite possible changes in carcass appearance due to variations in diet, growing condition, variety, etc. Strategies for using the equipment to screen the carcasses will be evaluated. Another objective will involve collecting carcasses with a variety of known diseases and defects, and determining which instrumentation is optimal for identifying each kind of disease and defect. Successful methods will be incorporated into the equipment developed in the first objective and tested on-line. Industrial prototype systems for poultry inspection will be assembled and tested in industrial processing environments for a prolonged period of time.

Summary Accomplishments During Entire Project:
An off-line experimental system that sensed poultry carcasses on moving shackles with a visible/near-infrared (Vis/NIR) spectrophotometer was assembled and measurements at a slaughter plant in West Virginia showed that average accuracies were above 94% for separating wholesome and unwholesome carcasses. The unwholesome carcasses included septicemia/toxemia (septox), cadaver, airsacculitis, ascites, and tumor. The highest average accuracy was obtained at 90 bpm (birds per minute) without room light (97.5%). The results also showed that the room light in the poultry processing plant was too low to impact the classification accuracy to any great degree; however, maintaining a consistent lighting environment could slightly improve the performance of the classification.

An on-line Vis/NIR chicken carcass inspection system was then assembled, and on-line trials were performed during an 8-day period in a commercial poultry slaughter plant. Spectra (470-960 nm) of 1750 (1174 wholesome and 576 unwholesome) chicken carcasses were measured. The instrument measured the spectra of veterinarian-selected carcasses as they passed on a processing line at 70 bpm. The detection time for each carcass was 0.3 seconds. The system was able to classify the carcasses with 95% accuracy. The results showed that the Vis/NIR spectrophotometer system is able to separate unwholesome from wholesome carcasses on-line in a poultry-processing environment.

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We have succeeded in applying the 2D correlation approach to systematically investigate the Vis/NIR spectral variations of chicken meats under a variety of conditions, such as thermal treatment, cold storage, thawing, wholesome and unwholesome. The results showed that four visible bands around 445, 485, 560, and 635 nm could be assigned to deoxymyoglobin (DeoxyMb), metmyoglobin (MetMb), oxymyoglobin (OxyMb), sulfmyoglobin (SulfMb), respectively. Their relative intensity changes are associated with the variation of meat color corresponding to external conditions.

We assembled a dual-camera spectral imaging system and successfully tested it on-line at ISL's pilot plant. On-line trials of this system in a commercial poultry-processing plant were conducted during a 14-day period, where spectral images of 13,132 wholesome and 1,459 unwholesome chicken carcasses were measured. The inspection system gave accuracies of 94% and 87% for wholesome and unwholesome carcasses, respectively. This accuracy was consistent with the results obtained previously on laboratory studies. Thus, the inspection system shows promise for separation of unwholesome chicken carcasses from wholesome carcasses in the poultry processing lines.

We used a color imaging system at ISL to classify viscera of wholesome and unwholesome carcasses. We collected in a poultry process plant color images of 320 livers and hearts from wholesome, airsacculitis, cadaver, and septox chickens. These images in red, green, and blue (RGB) color space were segmented and statistical analysis was performed for feature selection. A neuro-fuzzy system utilizing hybrid paradigms of fuzzy inference system was used to enhance the robustness of the classification processes. The accuracy for separation of wholesome from unwholesome livers ranged 87.5 to 92.5%. Classifying wholesome and unwholesome chicken hearts, the accuracies ranged from 92.5 to 97.5%.

We used multispectral image analysis techniques to characterize chicken heart images for disease detection. Spectral signatures of five categories of chicken hearts (wholesome, airsacculitis, ascites, cadaver, and septicemia/toxemia) were obtained from reflectance measurements taken with a Vis/NIR spectroscopic system in the range 474-974 nm. Multivariate statistical analysis was applied to select the most significant wavelengths for category separation. The multi-spectral image system has four narrow-band filters providing four spectrally discrete images on a single CCD focal-plane, using the four wavelengths selected from analysis of the reflectance spectra, (495, 535, 585, and 605 nm). The system was able to differentiate the five categories with accuracies of 92%, 100%, 96%, 84%, and 100%, respectively. The results show that multi-spectral imaging can provide a method of identify poultry diseases from multispectral images of viscera.

We conducted economic studies to evaluate the economic feasibility, value, and costs of using an automated inspection technology in place of visual organoleptic inspection. The results indicated that the economic benefits to FSIS would accrue from labor savings, whereas the economic benefits to slaughter plants would accrue primarily from increased throughput from faster inspection line speeds. The U.S. broiler industry would gain from $1.55-$2.57 billion revenue in current value over the next five years if automated inspection is used in place of organoleptic methods and line speeds are operated at 100 birds per min. The results also indicate FSIS could redeploy approximately 1342 inspectors to other in-plant tasks by adopting automated inspection, but would have to pay additional expenses related to the installation of the technology in slaughter plants.

These additional expenses range from $32 to $59 million in discounted cost over the five-year period, and could be payed either in part or in whole by the broiler industry.

Summary 2000 Accomplishments:
We systematically investigated the fundamental visible/NIR (Vis/NIR) spectral features of chicken meats. We studied the Vis/NIR spectral variations of chicken meats under a variety of conditions. The results showed that four visible bands around 445, 485, 560, and 635 nm could be assigned to deoxymyoglobin (DeoxyMb), metmyoglobin (MetMb), oxymyoglobin (OxyMb), sulfmyoglobin (SulfMb), respectively. Hyperspectral and multispectral imaging techniques were used to detect chicken skin tumors. A high spacial resolution hyperspectral imaging system was assembled, and its calibration technique was developed. Hyperspectral imaging techniques were used to detect chicken skin tumors. Hyperspectral images of eight tumorous chickens were taken in the spectral range of 420-850 nm. Principle component analysis was applied to select three optimal wavelength bands (465, 575, 705 nm) from the tumorous chicken images. A multispectral imaging system capable of simultaneously capturing three registered images was used to image 60 tumorous and 20 normal chickens. Multispectral image analysis was performed to generate ratioed images, which were then divided into regions of interest (ROI's) classified as either tumorous or normal by a veterinarian. Image features for each ROI were extracted for use as inputs to fuzzy classifiers. The fuzzy classifiers were able to separate normal from tumorous skin with increasing accuracies as more features were used. In particular, use of all three features gave successful detection rates of 91% and 86% for normal and tumorous tissue, respectively.

Project Research Accomplishments During Next 3 years:
Develop a second generation poultry inspection systems and initiate a CRADA for a commercial version of the poultry inspection system. Develop on-line calibration method for individual Vis/NIR spectrophotometer and multispectral imaging subsystems. Use laboratory Vis/NIR, FT-IR, FT-Raman, and fluorescence spectrophotometers to characterize spectra of parts of the carcass (such as meat, skin, tumors, etc.) to better understand the spectral phenomena on which our practical instruments are based. Conduct trials of an industrial-grade poultry inspection system prototype. Test a method that will use the online inspectors (called *sorters* under the HACCP-based Inspection Model Project) to provide the reference data for calibration modification in a way that will not disturb their normal routine. Collect carcasses and viscera with a variety of known diseases/lesions or defects, measuring them with several instruments available to the laboratory, and determining which instrumentation is optimal for identifying each condition. Develop detection algorithms for individual chicken diseases and defects. Conduct field testing of a commercial poultry inspection system. Design statistical process control protocols to evaluate the performance of the systems compared to veterinarians and line inspectors. Incorporate the algorithms for identifying individual conditions into the poultry inspection system. Examine optimal placement of the equipment along the processing line.

The program will be expanded in 2001 to include the inspection of plant produce with particular emphasis on apples. This will be reflected by the inclusion of the words "and Plant Produce" to the CRIS title. The specific goals of the new initiative will be: to develop techniques for the detection of fecal and soil contaminants, open cuts, bruises and fungal growth; and development and implementation of an on-line imaging system for real-time inspection.

Technology Transfer:
ISL scientists described the ISL automated poultry inspection system and presented a video showing the ISL poultry inspection system being tested at a poultry processing plant to FSIS managers including Mr. Mike Grasso, Dr. Charles Edwards, Dr. Haney Sidrak, Mr. Grey Berbano, Dr. Brij Bhargava, Mr. Hyder Lhakani, Dr. Howard Early, Dr. William James and Dr. Robert Brewer. Brief descriptions and videos showing and describing the operation of the ISL's Automated On-line Poultry Inspection System on-line at Tyson Foods, Inc. in New Holland, Pennsylvania were sent to Mr. Frank Nicoletti, Stork Gamco, Inc.; Mr. Grover Harben, Gainesville Processing Equipment, Inc.; Ms. Dee Franken, Johnson Food Equipment, Inc.; and Mr. Martijn Hillenius, MEYN Poultry Processing, LLC.

PUBLICATIONS:

Chao, K., Y.R. Chen, H. Early, and B. Park. 1999. Color image classification systems for poultry viscera inspection. Applied Engineering in Agriculture. Vol. 15(4): 363-369.

Chao, K., B. Park, Y.R. Chen, W.R. Hruschka, and F.W. Wheaton. 2000. Design of a dual-camera system for poultry carcasses inspection. Applied Engineering in Agriculture. Vol. 16(5): 581-587.

Chen, Y.R., W.R. Hruschka, and H. Early. 2000. A chicken carcass inspection system using visible/near-infrared reflectance: in plant trials. J. Food Process Engineering. Vol. 23(2): 89-99.

Liu, Y. and Y.R. Chen. 2000. Two-dimensional correlation spectroscopy study of visible and near-infrared spectral variations of chicken meats in cold storage. Applied Spectroscopy. Vol. 54(10): 1458-1470.

Liu, Y., Y.R. Chen, and Y. Ozaki. 2000. Characterization of visible spectral intensity variations of wholesome and unwholesome chicken meats with two-dimensional correlation spectroscopy. Applied Spectroscopy. Vol. 54(4): 587-594.

Liu, Y., Y.R. Chen, and Y. Ozaki. 2000. Two-dimensional visible/near-infrared correlation spectroscopy study of thermal treatment of chicken meats. J. of Agricultural & Food Chemistry. Vol. 48(3): 901-908.

Park B. and Y.R. Chen. 2000. Real-time dual-wavelength image processing for poultry safety inspection. J. of Food Process Engineering. Vol. 23(5): 329-351.

Tao, Y., J. Shao., K. Skeeles, and Y.R. Chen. 2000. Detection of splenomegaly in poultry with near-UV and color imaging. Trans. ASAE. Vol. 43(2): 469-474.

Watkins, B., Y.-C. Lu, and Y.R. Chen. 2000. Economic feasibility analysis for an automated on-line poultry inspection technology. Poultry Science. Vol. 79: 265-274.

PROCEEDINGS/ABSTRACTS:

Chao, K., Y.R. Chen, P.M. Mehl, and W.R. Hruschka. 2000. Automated tumor segmentation for poultry inspection. ASAE Paper No. 003084. ASAE, St. Joseph, MI.

7.6

CRIS Title: Development of Imaging Technology for the Automated On-Line Inspection of Poultry Products
CRIS: 6612-41420-030
Scientists: Windham WR, Lawrence KC, Smith DP, Lyon CE
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Summary Project Aims:
Several deaths occur each year from public consumption of contaminated poultry and/or meat. This contamination by bacterial microorganisms has led the public to require more careful inspection of meat and poultry. Such microorganisms are most commonly found in the digestive tract of the animals and their excreted feces. Potential contamination can occur when feces or ingesta is deposited on the surface of the carcass. A hyperspectral imaging system has been built and a method, which can be used for further real-time processing application, has been developed for fecal and ingesta detection on meat surfaces.

Summary Accomplishments During Entire Project:
An imaging system and method will be developed to provide a real-time method of detecting fecal & ingesta contaminates on poultry/meat carcasses. The invention will impact industry by providing a technique to detect sources of potential food safety contaminates, by reducing subsequent slaughter plant stoppages resulting from contaminated carcasses, and by reducing the water usage within the plant.

Summary 2000 Accomplishments:
A method and an imaging system for near real-time fecal and ingesta detection on meat carcasses was invented. The system developed consists of a diffuse fiber optic line light source and an associated hyperspectral imaging system comprising a high resolution CCD camera with a visible/NIR spectrograph, lens, associated optical hardware, frame-grabber, and computer which is capable of identifying location and size of poultry feces from poultry viscera (duodenum, colon,& ceca), and is independent of scald water temperatures (international patent disclosure pending). The system provides a basis for the development of a real-time fecal detection system for the poultry processing industry.

Projected Research Accomplishments During Next 3 Years:
In the first year, calibration protocols for the imaging system will continue to be developed and implemented. Protocols will be developed to monitor spectral noise, wavelength accuracy, and precision of the system. A control strategy for the line-scan camera to collect images that have sufficient spatial resolution and accuracy will be completed. Fecal detection algorithms will be validated from birds fed diets other than corn/soybean meal. A closed loop shackle system capable of running at commercial line speeds will be installed in a pilot-scale plant. In the second year, a real-time, portable hyperspectral and/or multispectral imaging system will be developed and installed on the shackle line. The systems and detection algorithms will be developed and tested to determine their performance on carcasses that are both artificially and naturally contaminated with feces and ingesta. The third year will see the research transferred to a commercial processing plant where on-line trial will be implemented that will test and validate the effectiveness of the system.

Technology Transfer:
An international patent disclosure is being prepared, Docket No. 0164.00 Method and System for Real-Time Fecal and Ingesta Detection During Meat and Poultry Inspection. A CRADA is currently being negotiated with an industry partner to transfer the technology, after in-depth discussions with two major poultry equipment companies have been held concerning detection and intervention to remove contaminants from poultry carcasses.

PUBLICATIONS:

Papers were prepared for presentation and publication; however, as ordered by our National Program Leader and the Office of Technology Transfer, all presentations and publications were withheld or withdrawn pending international patent disclosure.

7.8

CRIS Title: Engineering Innovations and Micro Developments to Reduce Contamination on Poultry Processing Equipment and Poultry
CRIS: 6612-41420-003
Scientists: Dickens JA, Buhr RJ, Cason JA, Hinton Jr. A , Lyon CE
Location: Poultry Processing and Meat Quality Unit, RRRC, Athens, GA
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Summary Project Aims:
Multi disciplinary and multi-faceted research projects from late grow-out through further processing to reduce or eliminate fecal contamination, and the associated contamination with human pathogens. Project areas include: feed withdrawal, transportation, pre and post-stunning, scalding, picking, evisceration techniques, chilling, and post-chill treatments. Procedures to limit the fecal matter in the lower gut of poultry brought into the plant or to void the lower gut during early phases of processing, antibacterial treatments to the drinking water during feed withdrawal, microbial modeling to predict the movement of microorganisms through the scalding equipment, and unique chemical treatments to kill or remove pathogens from processed carcasses are being investigated.

Summary Accomplishments During Entire Project:
A breeding population of Scaleless (featherless) chickens was established, to produce birds that will serve as a model for determining basic parameters of microbial contamination on the skin and to quantify intervention steps for their removal in comparison to feathered broilers. Computer models of bacterial survival in scald tanks indicated a complex pattern of multiple equilibria depending on the D values for different bacterial species. Computer modeling of water mixing during scalding demonstrates the movement and survival of bacteria in scald water can be predicted. Using the model it may be possible to predict the probability of cross-contamination of salmonellae to previously negative carcasses depending on numbers suspended in the scald water.

The effect of feed withdrawal on the physical, chemical, and microbial characteristics of the crops and ceca of turkeys was determined. Crop weight decreased significantly and crop pH increased significantly during feed withdrawal. The number of aerobic bacteria and Enterobacteriaceae in the crop increased while the number of lactic acid bacteria in the crop decreased during 24 h of feed withdrawal. Decreases in the number of LAB and increases in the number of aerobes and Enterobacteriaceae were found in the ceca of turkeys subjected to feed withdrawal. Broilers and turkeys were put on strict feed withdrawal regimes and the force to shear specific intestinal segments was studied. Contrary to previous subjective evaluations, longer feed withdrawal times did not lower intestinal strength. The force required to break intact and excised esophageal tracts from broilers demonstrated that the crop is the inherent weak segment of the alimentary tract.

Broilers transported and held on standard solid flooring had noticeably dirtier feathers and higher coliforms and E.coli counts prior to scalding and picking, but bacterial recovery from external carcass rinses did not differ between the solid and wire flooring treatments after feather removal. These results indicate that initial carcass bacterial load upon entering the processing plant does not directly correlate with carcass bacterial load after feather removal. Experiments comparing electric and carbon dioxide stunning and immersion and spray scalding demonstrated that spray scalding with carbon dioxide stunning reduced both coliforms and generic E. coli by marginal amounts.

Carcasses were electrically stimulated with 200 volts alternating current to force defecation of the carcasses prior to entering the scalder. 87% of the carcasses voided the lower gut during the stimulation. Unpublished data from a cooperating processor showed a 50% reduction in reprocessing when the carcasses were electrically stimulated. Adoption of this research would lead to fewer carcasses being contaminated with feces thus reducing the associated pathogen contamination. A secondary advantage of this equipment allows post chill ageing time to be reduced without adversely affecting tenderness. The electrical stimulator is being used in more than 40 commercial processing plants. Microbiological sampling of a three-tank, counter-flow scalder operating in a commercial processing plant showed that numbers of aerobic bacteria, coliforms, and E. coli decrease significantly in successive tanks. Salmonellae were isolated from scald water less frequently and at lower concentrations in the final tank, indicating that cross-contamination by salmonellae is less likely in a counterflow tank. Subcutaneous temperature profiles were done to compare immersion and spray scalding a various temperatures to determine the feasibility of spray scalding. Subcutaneous temperatures were monitored for either two minutes in immersion scalders at 52 and 56 C or for 30 sec to 1 min at three spray scalding temperatures (60, 65, 70 C). 70C for 30 seconds proved to give a comparable temperature profile and a comparable pick. Spray scalding, if accepted, could reduce or eliminate the cross-contamination now associated with standard immersion scalding.

Crop breakage occurring during evisceration has the potential to contaminate the carcass with enteric pathogens that are found in the crop ingesta. A new method of pulling the crop where the spine is severed prior to crop removal demonstrated significantly more crops could be removed intact. This process could significantly lower the contamination of broiler carcasses with the ingesta as well as associated pathogens. Crop peak extraction force and the incidence of ruptured crops of broilers was determined as influenced by the parameters: age, gender, and extraction direction; electrical stunning voltage (12, 50, and 200 V AC) and postmortem electrical stimulation; feed withdrawal duration and head removal prior to picking. Extraction of the crop toward the head consistently resulted in a higher incidence of intact crops (> 91%) than conventional extraction through the thoracic inlet (59 to 72% intact). The presence or absence of cutaneous nerve innervation does not appear to influence feather retention force (FRF) ante- or post-mortem. This finding indicates that treatments disabling the central nervous system ante-mortem may lower feather retention force indirectly by altering cutaneous metabolism. Previously reported work demonstrated that post-mortem FRF was unaffected by spinal cord severing immediately following stunning. These results reverse previously held industry beliefs that the spinal cord should remain intact until after death. Determined that oleic acid is bactericidal in vitro to pathogenic and spoilage bacteria associated with processed poultry carcasses. Populations of S. typhimurium, L. monocytogenes, C. jejuni, P. aeruginosa, and E. coli were reduced or completely eliminated when exposed to oleic acid. Incorporating an oleic acid wash into poultry processing plants could reduce the number of human human pathogens and extend the shelf-life of fresh poultry products.

Summary 2000 Accomplishments:
A provisional patent application was submitted for a cocktail that reduces the number of foodborne pathogens in the crop of broilers subjected to feed withdrawal. Commercial compounds (Protecta I, II, III a&b) were tested at various concentrations either in vitro or on poultry and poultry parts to determine its effect on the microbiological quality. Concentrations as low as 0.1% killed Listeria in vitro whereas up to a 3% for short contact periods were required when used on poultry products.

Solutions at 1 and 2% in the chill water all but eliminated pathogenic bacteria and reduced other bacteria to levels below accurate detection limits. The recovery of Campylobacter from the hen's reproductive tract, from the cloacal to the magnum, indicated the possibility of direct vertical transmission of this pathogen from hen to egg to broiler chick. When the crop is removed toward the head or the neck removed prior to extraction through the thoracic inlet, >92% of crops are removed intact during manual evisceration. Crops that rupture during removal required grater maximum load, indicating the crops break due to greater adhesions not due to weakness.

Projected Research Accomplishments During Next 3 Years:
Establish an MOU with a poultry integrator to continue evaluation of the cocktail under commercial conditions. Evaluate the influence of the duration of feed withdrawal and cooping time on carcass microbial load when entering the processing plant. Cross the F1 population of Scaleless X Broiler hybrids chickens, to produce featherless and broilers that are similar in body weight. The F2s will serve as a model for determining location and attachment of bacterial pathogens on the skin and to quantify intervention steps for their removal in comparison to de-feathered broilers. Bacteria in feather follicles will be studied to determine the role of the follicles in overall bacterial contamination. Bacteria will be counted on feathered and unfeathered areas of breast skin before and after scalding and picking. Histology samples of the follicles will determine the depth of penetration of bacteria in the follicle. A technique for continuous rinsing and sampling of broiler carcasses will be used to determine the patterns by which different bacteria are removed from poultry carcasses when the carcasses are in water. Utilize the surgical evisceration technique to permit accurate determination of internal and external carcass microbial contamination during scalding and de-feathering steps of commercial processing. Evaluate alternative evisceration techniques to remove the alimentary tract intact while maintaining attachment to the spinal column. Examine the bactericidal activity of other fatty acids in vitro and on carcasses of processed broilers. Examine the prevalence of yeasts in poultry processing operations. Determine the optimum parameters and application techniques for use of the herbal extract for microbiological quality, shelf life extension and economics for processed poultry. Determine the effect of electrical stimulation on the gastrointestinal tract of broilers initiated this year will be completed along with meat quality studies.

Technology Transfer:
Filed a patent application for a process for rapid tenderization of poultry breast meat. Approved but not issued. At least 40 processors in the United States and one processor in Brazil have installed electrical stimulation equipment in their plants based on design work supplied by this unit. Installation of a stimulator in a large hen processing plant (Crider Poultry, GA)is under way as well as with a broiler processor (Pilgrim's Pride, TX) where paired comparisons will be possible for both tenderness and reduced fecal contamination. Simmons Engineering, Dallas, GA has built an electrical stimulation unit based on design work supplied by this unit, and has it commercially available to the poultry industry. Simmons has commercialized the automated microbiological whole carcass rinse shaker and have sold several units to industry. A patent disclosure has been filed for a procedure to treat commercial hatching eggs to eliminate salmonellae from commercial hatchlings. A provisional patent application was submitted for a cocktail that reduces the number of foodborne pathogens in the crop of broilers subjected to feed withdrawal, and a MOU is being developed with Sanderson Farms, MS, to commercially test the cocktail. Scientists are using the methodology developed by CRIS scientists to evaluated feather retention force as influenced by soft

scalding in broilers. Sternum bone compression strength values of broilers as influence by age and gender has been transferred to equipment manufacture Stork GAMCO to aid in the development of mechanical breast filleting equipment.

PUBLICATIONS:

Dickens, J.A., Berrang, M.E., Cox, N.A. Efficacy of an herbal extract on the microbiological quality of broiler carcasses during a simulated chill. 2000. Poultry Sci. v.79(8). p.1200-1203.

Hinton, Jr.,A., Buhr, R.J., Ingram, K.D. Physical, chemical, and microbiological changes in the crop of broiler chickens subjected to incremental feed withdrawal. 2000. Poultry Sci. v. 79(2). p. 212-218.

Hinton, Jr.,A., Buhr, R.J. ,Ingram, K.D. Physical, chemical, and microbiological changes in the ceca of broiler chickens subjected to incremental feed withdrawal. 2000. Poultry Sci.v. 79(4). p. 483-488.

Hudson, H.A., Wilson, J.L., Rowland, G.N., Buhr, R.J., Britton, W.M. Feed restriction affects bone properties of the broiler breeder pullet femur. 1999. J. of Appl. Poultry Res. v. 8(4). p.400-407.

Northcutt, J.K., Smith, D.P., Buhr, R.J. Effects of bruising and marination on broiler breast fillet surface appearance and cook yield. 2000. J. of Appl. Poultry Res. V.9(1). p.21-28.

PROCEEDINGS/ABSTRACTS:

Buhr, R.J.,Cason, J.A., Dickens, J.A., Marshall, D.E. Influence of pre-evisceration carcass trimming and extraction direction on crop extraction force and efficiency of crop removal during manual evisceration of broilers. 2000. Poultry Sci. v. 79(Suppl.1):Abstract p. 103.

Buhr, R.J., Dickens, J.A., Pizzino, D.R. Influence of feed withdrawal and stunning voltage on crop extraction force and efficiency of crop removal during manual evisceration of broilers. 2000. Poultry Sci. v. 79(Suppl.1):Abstract p. 104.

Buhr, R.J., Cox, N.A., Stern, N.J., Wilson, J.L. Recovery of Campylobacter jejuni from segments of the reproductive tract of broiler breeder hens. 2000. Poultry Sci. v. 79(Suppl.1):Abstract p. 112.

Buhr, R.J., Dickens, J.A. Influence of gender, age, and direction of extraction on peak pull force and the incidence of crops removed intact from broilers during manual evisceration. 2000. Poultry Sci. v. 79(Suppl.1):Abstract p.59.

Cason, J.A. Distribution and survival of bacteria during poultry scalding. Proceedings, 14th European Symposium on the Quality of Poultry Meat, Bologna, Italy, September 19-23, 1999. V. 1. p. 359-363.

7.12

Cason, J.A., Berrang, M.E. Bacterial counts on right and left sides of pre-chill broiler carcasses. 2000. Poultry Sci. v. 79(Suppl.1):Abstract p. 105.

Hinton, Jr., A.,Ingram, K.D. Influence of oleic acid on populations of Campylobacter on poultry skin and in vitro. 2000. Poultry Sci. v. 79(Suppl.1):Abstract p. 102.

CRIS Title: Reduction & Control of Pathogens Associated with Food Processing Surfaces
CRIS: 6612-41420-006
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Summary Project Aims:
The phenomenon of bacterial attachment to processing plant surfaces such as metals, rubber, and plastics presents a formidable obstacle for sanitizing and cleaning treatments. When bacterial cells initially attach to a surface, they can produce extracellular polymers that anchor the cells and provide a favorable environment for growth and the subsequent attachment of more bacteria, other microbes and debris. The composite is a biofilm that is resistant to cleaners and sanitizers and is extremely difficult to remove. Food safety could be enhanced by increasing the use of materials that do not support growth and attachment of microorganisms while decreasing the use of materials that enhance growth and attachment. Inhibiting bacterial attachment will enhance food safety by preventing the increase in bacterial numbers necessary for biofilm formation. Finding the least amount of treatment necessary to effectively inhibit biofilms will be economical for the industry and consumers as well as reduce the impact of agriculture on the environment.

Summary Accomplishments During Entire Project:
Methods were developed to measure attached bacteria and biofilm formation on surfaces, to identify materials that were resistant to attachment from an array of surface materials, and to develop and compare physical and electrochemical treatments of stainless steel for inhibition of bacterial attachment and biofilms. The research provided the national food safety program and the processing industry with new information on bacterial attachment to processing plant surfaces and has shown that food safety could be enhanced by using materials that do not support bacterial growth and attachment. As requested by cooperators, authorized by Administration and NPS, a research project was conducted jointly with the University of Georgia, Food Safety and Inspection Service, and Gold Kist scientists to test the efficacy of new on-line technology for reduction of fecal contamination on broiler carcasses. Equipment for on-line processing of visually contaminated carcasses was tested and reduced the number of carcasses being subjected to off-line reprocessing. There were no significant effects from on-line treatment for aerobic plate counts, Salmonella, Campylobacter or coliforms. This research was one of the first in the world to use digital aroma technology to detect bacterial contamination and classify microorganisms. This method had been traditionally used in the food and flavor industry to detect off-odors. Bacterial species important to poultry processing as potential pathogens were isolated, and the technology was put to novel use to compare the isolates and profile the characteristics of the microbial populations. Profiles of pathogens such as Salmonella, E.coli, and Listeria were compared with plant isolates by digital aroma technology, SPME, and mass spectroscopy. The metabolic processes of microorganisms growing on chicken meat surfaces were examined with the ultimate goal of controlling these processes in order to yield safer poultry products with less potential for spoilage.

Substrate utilization data obtained in this research may be useful in characterizing and controlling biofilm communities.Reduction or removal of rapidly metabolized substrates, for example, in the poultry processing environment may reduce biofilm formation and persistence. Candidate substrates

may include some of the 17 individual substrates identified in this study whose frequency of utilization differed by greater than 30% between biofilm communities associated with meat samples stored at 4°C or at 13°C. Changes in the measurements of roughness parameters for treated stainless steel surfaces by atomic force microscopy (AFM) corresponded with the reduction of bacterial contamination and early biofilm formation shown by scanning electron microscopy (Dr. George Bailey, EPA, collaborator). Therefore, AFM may be a useful tool to predict the potential for bacteria to attach and form biofilms on other surfaces. These new findings will aid equipment manufacturers in selecting materials and finishes that are not conducive to bacterial growth and biofilm formation. The design of appropriate materials for the reduction of biofilms during food processing necessitates an understanding of the forces of bacterial attachment and biofilm formation. To understand these processes will enable us to develop interventions to enhance plant sanitation practices and pathogen control. In future work, the AFM might also be used to determine the importance of surface topography and chemistry on the diversity of pathogenic microbes associated with surface biofilms.

Summary 2000 Accomplishments:
The reduction of bacterial attachment to stainless steel and other surface materials in the food processing environment would enhance sanitation and pathogen control. A project with a university, sister agency, poultry processor, and two equipment companies established rapid methodology for characterization of surfaces to determine bacterial resistance. Factors that are important for resistance of stainless steel surfaces, the most common in food processing, to bacterial contamination were determined. The new findings, related to the bacterial resistance of treated stainless steel and other materials, are being used to set standards for equipment surfaces in the meat and poultry industries.

An understanding of the microbial ecology of biofilm communities associated with poultry products is of importance with respect to pathogenicity and food quality. The substrate utilization profiles of bacterial communities (biofilm) were assessed as they developed on chicken meat samples stored for varying time periods at two temperatures commonly used in a poultry processing facility. Metabolic capabilities of these mixed microbial populations will allow more efficient intervention strategies to be developed for use in poultry processing environments. In poultry processing environments, removal or reduction of the concentration of substrates readily metabolized by bacterial communities (biofilm) which develop on meat or processing equipment surfaces may decrease contamination of product by decreasing biofilm formation and persistence. Effective control or intervention measures to improve sanitation practices for bacterial contamination in processing facilities are needed. This problem has been addressed by studying the efficacy and mechanisms of potential chemical agents selected from several groups of substances that destroy or limit microbial growth on food surfaces or inanimate objects under a CRADA with Ajay North America, LLC (Dr. Ida Yates, ARS, co-PI) and Trust Agreement with NVID Corp. The resistance of a number of bacterial species most commonly associated with food-borne illness and food spoilage in the poultry processing environment were measured and compared. The ideal product formula would be non-toxic to humans and have the greatest efficacy against pathogenic bacteria, the least impact on the environment, and the lowest cost for the poultry processing industry.

Because of the successful application of digital aroma technology (the electronic nose) to the assessment of the quality of other food products, the potential existed for application of this technology to poultry products. The electronic nose was used to assess poultry meat as a function of storage temperature and time. The nose could detect differences in number of days that poultry meat was stored and distinguish between meat that had been properly refrigerated and meat that had been allowed to warm and then recool, as well as distinguish between meat products that were processed differently. Versions of the electronic nose will be useful in industry, food processing, medicine, national security, and the home. Preliminary testing of technology to transfer a strong negative electrostatic charge to biofilms on stainless steel has shown promise to reduce bacterial contamination on surfaces. A small chamber with an electrostatic space charge system was used to treat mixed bacterial populations from the poultry processing environment that were attached to stainless steel coupons (Dr. Bailey Mitchell, ARS, collaborator). The negative air ionization system effectively decreased the survival levels of bacteria on stainless steel coupons to the same as negative controls outside the system. This technology could have applications that extend into other food processing areas, medical institutions, and the home.

Projected Research Accomplishments During Next 3 Years:
Continue test development with analysis of pathogen impact. Continue to test specific compounds for efficacy of biocidal action in laboratory studies. Continue testing surface materials. Isolate and characterize resistant bacteria. Continue test development with analysis of pathogen impact. Begin field studies to test compounds and surface materials. Test inhibitory compounds for longevity of action and surface corrosiveness. Continue characterization of resistant bacteria. For FY 2003, continue field studies to determine most effective combinations of compounds and materials in processing plants. The resistance of mixed bacterial populations, and biofilms to recently developed disinfectants and sanitizers will be tested. Combining the surface material most resistant to bacterial attachment with cleaning by the most effective agent will maximize the potential of this research to both enhance food safety and reduce the impact of sanitation practices on the environment.

Technology Transfer:
Technologies listed in the accomplishments have been transferred to the Extension Service, action agencies, universities, equipment manufacturers, and the poultry industry through invited lectures, written reports, and scientific meetings. A CRADA with a manufacturer seeks to develop compounds suitable for use in preventing growth and controlling contamination by microorganisms on minimally processed food and feed products. A Trust Agreement with a manufacturer seeks to develop an application for an alternative disinfectant for chlorine during poultry processing.

PUBLICATIONS:

Arnold, J.W., Bailey, G.W. Comparison of scanning electron and atomic force microscopy of surface finishes on stainless steel that reduce bacterial attachment. Scanning, Journal of Scanning Microscopy. Mar 2000. v. 22 (2). p. 115-117.

7.16

Arnold, J.W., Silvers, S. Comparison of poultry processing equipment surfaces for susceptibility to bacterial attachment and biofilm formation. Poultry Science. Aug 2000. v. 79 (8). p. 1215-1221.

PROCEEDINGS/ABSTRACTS:

Arnold, J.W., Boothe, D.D. Comparison of poultry meat at 4C and 13C by digital aroma technology (electronic nose). Poultry Science. 2000. 79(Suppl.1): Abstract p. 104.

CRIS Title: Microbial Ecology and Transmission of Human Pathogens during Poultry Processing
CRIS: 6612-41420-007
Scientists: Meinersmann RJ, Berrang ME, Lyon CE
Location: Poultry Processing and Meat Quality Unit, RRC, Athens, GA
Contact: 706-546-3236 (P); 706-546-3633 (F); rmeiners@saa.ars.usda.gov

Summary Project Aims:
Poultry products can serve as a source for human infection by bacteria that can cause diarrheal disease. Poultry products undergo many manipulations in the processing facility that could affect the microbial quality of the final product. The ultimate goal of this research is to lower bacterial contamination and incidence of foodborne pathogens on processed poultry by studying microbial interactions within the poultry processing environment. The specific objectives to achieve this goal are: Define the sources and transfer points of human pathogens including Campylobacter spp. and Listeria monocytogenes within poultry processing. Trace the sources and adaptations of human pathogens and examine places harboring the pathogens by sampling the poultry-processing plant ecosystem and applying genetic technologies.

Summary Accomplishments During Entire Project:
This project was newly instituted last year; the accomplishments in the past year represent the life of the project.

Summary 2000 Accomplishments:
We need to be able to globally trace the distribution of Campylobacter jejuni to understand its ecology. A large population of the organism was evaluated using population genetic techniques. It was discovered that the bacterial species C. jejuni exists in two types, one that readily shares DNA among other members of the species and a type that does not. The type that do not readily share DNA were not found in chicken samples and did include most of the strains associated with Guillian-Barre Syndrome. It had earlier been noted that bacterial counts on broiler carcasses decrease during scalding and increase again during defeathering. However, the addition of a second hot water treatment following defeathering did not lower bacterial counts on broilers. Part of the reason for these results is that without feathers the carcass can not be treated with water as hot as the original scald tank. Although a substantial number of Campylobacter cells are found on skin of broilers during processing, the numbers found on store bought product without skin were found to be no different than paired product with skin. However, a difference was noted between partially processed parts with and without skin, indicating that the unavoidable compromising of skin associated with evisceration allows bacteria (including Campylobacter) to come into contact with broiler meat below the skin. This information may be used to test novel processing procedures to minimize the contamination of meat with skin bacteria. We have begun the preparation of hybridization arrays for the study of Campylobacter physiological responses.

Projected Research Accomplishments During Next 3 Years:
Design and test techniques for potential to decrease Campylobacter found on poultry products. This will be addressed as potential techniques become apparent and will be conducted through out the term of the CRIS as appropriate from year one to year three. Create or acquire a marker strain of Campylobacter for use in experiments. Validate current sequence-based subtyping for tracking of

isolates of Campylobacter. Create genomic library of Campylobacter for use in gene expression studies. Determine the extent that a) carcass to carcass and b) colon to surface contamination alone can increase the number of Campylobacter associated with carcasses as they move through the picker room. Determine of the effect of each major processing step on the population of L. monocytogenes on carcasses and/or product and identification of the primary problem areas.

Determine the extent that a) air contamination and b) picker finger contamination alone can increase the population of Campylobacter associated with carcasses as they move through the picker room. Evaluate the population genetics of Campylobacter. Multi-locus sequence typing of populations will be performed to determine if clones of Campylobacter can be linked to the source from which they are isolated. Determine correlation of sub-types of Campylobacter entering a processing plant on a bird with the types that leave the plant on the product using highly discriminatory sub-typing. Determine antimicrobial resistance transmission within the poultry processing plant. Determine if sub-types of bacteria leaving a processing plant have gained or lost antimicrobial resistance. Develop method to synchronize growth of Campylobacter cultures and monitor expression of genes in different phases of growth. Monitor gene expression by Campylobacter recovered from various microhabitats found within a poultry processing plant.

Examine of further processing facilities for presence of L. monocytogenes at different stages, determine if a relationship exists between primary processing incidence and further processing incidence. If there are changes in the types of organisms entering and leaving the plant, repeat determinations using isolates gathered at multiple points through processing.

PUBLICATIONS:

Berrang, M.E., Buhr, R.J., Cason, J.A. Campylobacter recovery from external and internal organs of commercial broiler carcass prior to scalding. Poultry Science. Feb 2000. v. 79. p. 286-290.

Berrang, M.E., Frank, J.F. Buhr, R.J., Bailey, J.S., Cox, N.A. Eggshell membrane structure and penetration by Salmonella Typhimurium. Journal of Food Protection. Jan 1999 v. 62. p. 73-76.

Berrang, M.E., Cox, N.A., Frank, J.F., Buhr, R.J. Bacterial penetration of the eggshell and shell membranes of the chicken hatching egg: a review. Journal of Applied Poultry Research. Winter 1999. v. 8. p. 499-504.

Cox, N.A., Berrang, M.E., Buhr, R.J., Bailey J.S. Bactericidal treatment of hatching eggs II. Use of chemical disinfectants to reduce Salmonella on hatching eggs. Journal of Applied Poultry Research. Fall 1999. v. 8. p. 321-326.

Kapczynski, D.R., Meinersmann, R.J., Lee, M.D. Adherence of Lactobacillus to Intestinal 407 Cells in Culture Correlates with Fibronectin Binding. Current Microbiology. 2000. v.41(2). p. 136-141.

Locke D.P., Sellers H.S., Crawford J.M., Schultz-Cherry S., King D.J., Meinersmann R.J., Seal B.S. Newcastle disease virus phosphoprotein gene analysis and transcriptional editing in avian cells. Virus Research. Sept 2000. v. 69(1). p. 55-68.

Meinersmann, R.J., Hiett, K.L. Concerted evolution of duplicate fla genes in Campylobacter. Microbiology. Sept 2000. v.146(9). p. 2283-2290.

Meinersmann, R.J. Population genetics and genealogy of Campylobacter jejuni. Nachamkin, I. and Blaser, M.J., editors. ASM Press, Washington DC. Campylobacter, 2nd Edition. Jan 2000. p. 351-368.

Seal B.S., Sellers H.S., Meinersmann, R.J. Fusion protein predicted amino acid sequence of the first US avian pneumovirus isolate and lack of heterogeneity among other US isolates. Virus Research. Feb 2000. v. 66(2). p. 139-47.

Seal B.S., King D.J., Meinersmann, R.J. Molecular evolution of the Newcastle disease virus matrix protein gene and phylogenetic relationships among the paramyxoviridae. Virus Research. Jan 2000. v. 66(1). p. 1-11.

Wassenaar, T.M., On, S.L.W., Meinersmann, R.J. Genotyping and the consequences of genetic instability. Nachamkin, I. Blaser, M.J., editors. ASM Press, Washington DC. Campylobacter, 2nd Edition. Jan 2000. p. 369-380.

PROCEEDINGS/ABSTRACTS:

Berrang, M.E., Ladely, S.R. Presence and level of Campylobacter on broiler parts with and without skin. Poultry Science. Jul 2000 v. 79(s1). p. 103.

Berrang, M.E., Dickens, J.A., Musgrove, M.T. The effect of hot water treatments after defeathering on the microbiological contamination of broiler carcasses. Poultry Science. Jul 2000. v. 79(s1). p.103.

Berrang, M.E., Ladely, S.R. Effect of pre-chill skinning on the level of Campylobacter recovered from broiler parts. Proceedings 87th Annual meeting of International Association of Food Protection. Aug 2000. p. 99.

Musgrove, M.T., Berrang, M.E., Byrd, J.A., Stern, N.J. Detection of Campylobacter spp. in ceca and crops with and without enrichment. Poultry Science. Jul 2000. v. 79(s1). p. 90.

7.20

CRIS Title: Effects of Processing Treatments on Safety & Quality of Raw and Cooked Poultry Products
CRIS: 6612-41420-005
Scientists: Lyon CE, Jones DR, Young LL
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Summary Projects Aims:
The charge for this team is to optimize processing efficiency and ensure end-product quality and safety. The CRIS addresses both safety and quality issues. Three problems/issues are being addressed:1) modify processing parameters for maximum efficiency and quality and 2) optimize processing operations to meet market direction and 3) develop markers to accurately note the heat treatment applied to poultry meat. Research is focused on optimizing processing, further-processing and packaging of intact parts and fabricated products, with emphasis on breast tissue since it is the economic driver of the industry. Specific objectives are to optimize yield, texture and color of these parts/products by coordinating production practices such as age at slaughter; postmortem processing factors such as state of rigor and marination techniques; and storage and shipping operations such as minimum weight packaging. Development of methods to identify markers that can be used to determine the heat treatment that has ben applied to poultry meat is essential for both industry and regulatory personnel.

Summary Accomplishments During Entire Project:
Cooperative research with the Quality Assessment Research Unit resulted in texture benchmarks for cooked breast meat. The relationship between objective procedures and sensory panels was established. Ranges of tough to tender for each procedure were related to a sensory scale. Objective values relating to the tender portion of the scale are routinely used by industry quality control personnel to ensure process control and product quality. An in-line apparatus conceived, built, and tested under pilot plant and commercial conditions applies pulsed electric current to carcasses to hasten postmortem biochemical reactions (deplete energy from the fiber) thus making it feasible to remove the large breast muscles without the traditional 6 hour aging time. With stimulation, the aging time can be reduced to 2 hours. Research established that the onset of rigor mortis could be hastened by subjecting carcasses to pulsed electrical stimulation during bleeding, and that marination of the breast muscles after carcass chilling resulted in acceptable tender breast meat without any further aging. This stimulation/marination treatment allows central preparation of breast meat products at the processing facility in a continuous flow which increases efficiency and reduces costs and handling. It is estimated that the cost savings will be $5,000,000 per plant each year. Various enzyme based systems were developed to determine adequacy of heating poultry and red meat. Examples include the GOT, PK, and ACP tests. The tests have proved to be accurate to the point that they are being used by both regulatory and industry personnel as integral parts of their Hazard Analysis Critical Control Point (HACCP) approach to science-based inspection. Cooperative research within the unit resulted in a patented process to tenderize breast meat by removing it from carcasses shortly after slaughter and placing in a clamp to prevent fiber contraction and subsequent toughening. The technique may allow breast meat to be removed earlier in processing instead of aging whole carcasses after chilling (patent approved, not yet issued).

Summary 2000 Accomplishments:
Yield studies in cooperation with University of Georgia faculty showed that bird age has a significant impact on the resultant proportion of parts. Even as feed efficiency declines as age increases, the proportion of meatier parts increases. Moreover, age also affects texture of early-harvested breast fillets. Early-harvested breast meat from younger birds was more tender than that of older birds. This information allows processors to designate specific marketing avenues based on bird age while minimizing process changes. Studies established that significant losses due to package overfilling of breast fillets could be reduced by initially weight categorizing the fillets then filling the packages by count instead of absolute weight. This results in a finite and known proportion of underweight packages, and that proportion can be controlled within tolerable levels.

Projected Research Accomplishments During Next 3 Years:
The impact of processing variables such as temperature, deboning time, marinade composition, vacuum, meat mass, and marination time on ultimate product quality will be developed for various types of meat/parts. Distribution of end-point temperatures within populations of cooked products will be modeled. Proportion of undercooked products will be estimated from the models under various processing protocols. Unintended changes in the processing protocols such as line speed interruptions, piece size variation, and ingredient changes will be added to the models. Studies will be conducted to optimize clamping conditions for broiler breasts. Applying pulsed electric current during clamping will be evaluated to determine if the time can be shortened by speeding the rate of postmortem ATP depletion. New approaches will be explored to develop markers that accurately determine the heat that has been applied to poultry meat. Optimum electrical stimulation conditions will be established to speed rigor development and void the lower gastrointestinal tract of fecal material. Markers that can replace temperature readings will be evaluated under commercial conditions to establish versatility and accuracy.

Technology Transfer:
The specifics of the apparatus designed to stimulate carcasses during bleeding have been transferred to domestic poultry companies and is either in use or is being tested in at least 24 plants and a company in Brazil. An equipment company, Simmons Engineering, Dallas, Ga., has used the concept to market a commercial version of the stimulator. Many of the processing companies are making their own in-house version of the stimulator based on information provided.

PUBLICATIONS:

Cason, J.A., Lyon, C.E., Dickens, J.A. Process for the rapid tenderization of poultry breast meat deboned immediately after evisceration. 2000. Patent approved, not yet issued. Invention Report #0128.94.

Lyon, C.E., Buhr, R.J. Biochemical Basis of Meat Texture. In: Poultry Meat Science, Poultry Science Symposium Series, ed. R.I. Richardson, G.C. Mead, CABI Pub., 1999. Oxfordshire, Eng. p. 99-126.

Young, L.L., Buhr, R.J., Lyon, C.E. Effect of polyphosphate treatment and electrical stimulation on post-chill changes in quality of broiler breast meat. Poultry Science. 1999. v. 78 (2). p. 267-271.

Young, L.L., Northcutt, J.K. Poultry Processing. In Food Proteins: Properties and Applications, ed. S. Nakai, H.W. Modler, John Wiley, 1999. New York, NY, USA. p. 147-169.

PROCEEDINGS/ABSTRACTS:

Northcutt, J.K., Lyon, C.E., Buhr, R.J., Young, L.L., Poole, G.H., Dickens, J.A., Alley, M., Bilgili, S.F., Hess, J.B. Effect of age, gender and commercial source of broilers on yield of carcass parts. Poultry Science. 1999. v. 78 (Suppl. 1). Abstract p. 123.

CRIS Title: Adhesion and Control of Human Pathogens to and on Surfaces: (Part A: Poultry)
CRIS: 5325-42000-022
Scientists: Mandrell RE, Charkowski AO, Cooley MB, Friedman M, Gorski L, Kint S, Miller WG
Location: Food Safety and Health Unit, WRRC, Albany, CA.
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Summary Project Aims:
Bacterial contamination of poultry is an increasing problem in the United States, and recognition of this problem by the American public is gaining. However, very little is known about how pathogenic bacteria exist in food environments. Both basic and applied research is needed to increase our understanding of how these pathogens attach to and survive in environments related to food, including meat, and processing environments. Our work has concentrated on the development of: (a) new detection methods for the identification of pathogens on foods; (b) attachment models to better understand the attachment process of several pathogens to food surfaces, including poultry skin (c) screening for natural antimicrobials that may be added to foods; and (d) the development of new strategies to prevent or minimize bacterial contamination during growth and processing.

Summary Accomplishments During Entire Project:
We have developed reagents for the detection of Campylobacter in foods; stable fluorescent Salmonella, Campylobacter, E. coli O157:H7, Listeria monocytogenes and plant bacterial strains for studies of the bacterial ecology and biology in complex environments and biofilms; and attachment assays to study pathogen interactions on surfaces. These assays will provide a quantitative means to measure pathogen reduction, and will enable the characterization of specific gene products that can be targeted for anti-adherence strategies. In addition, our work on biofilms should lead to a more thorough understanding of the natural attachment process of pathogens. We anticipate that these studies will lead to the identification of non-toxic methods to decrease pathogens in real food environments.

Summary 2000 Accomplishments:
Strains of C. jejuni and Salmonella containing plasmids that encode stable fluorescent proteins have been constructed and used in studies of attachment of bacteria to chicken skin and to factors isolated from skin. Proteoglycans have been identified as a candidate attachment factor for Campylobacter jejuni on chicken skin. Fluorescent bacteria have been fed to chickens to determine where the pathogens concentrate in the gastrointestinal tract, whether they change after passage, and to compare fitness of different strains. Initial results of Salmonella enterica and Campylobacter jejuni co-inoculation studies indicate intra- and inter-species competition and/or exclusion may occur at certain sites in the gastrointestinal tract.

Projected Research Accomplishments During Next 3 Years:
Identify chicken skin attachment factors. Determine sites of colonization of Campylobacter in chicken gastrointestinal tract. Characterize Campylobacter signaling pathways and relationship to biofilm formation and attachment. Identify plant antimicrobials useful for minimizing pathogens in poultry. Determine whether pathogen contamination pre-harvest increases resistance of pathogen

to interventions. Develop improved methods for isolating multiple pathogens from naturally contaminated poultry using new reagents and knowledge gained from basic studies.

Technology Transfer:
Technology transfer is an active part of the current CRIS project. Information and technology will be transferred to industry, government regulators, and to the scientific community via publications, collaborations, CRADA's, and Trust Agreements with industry. We continue to seek partners for licensing anti-pathogen antibodies produced in our labs.

PUBLICATIONS:

Kimura, R., Mandrell, R.E., Galland, J.C., Hyatt, D., Riley, L.W. Restriction-site-specific PCR as a rapid test to detect enterohemorrhagic Escherichia coli O157:H7 strains in environmental samples. Applied Environmental Microbiology 2000; v. 66(6). p.2513-9.

Alfano, J. R., Charkowski A. O., Deng W. L., Badel J. L.,Petnicki-Ocwieja, T.,van Dijk, K.,Collmer, A. The Pseudomonas syringae Hrp pathogenicity island has a tripartite mosaic structure composed of a cluster of type III secretion genes bounded by exchangeable effector and conserved effector loci that contribute to parasitic fitness and pathogenicity in plants. Proceedings of the National Academy of Science USA. 2000. v. 97(9). p.4856-61.

Collmer, A., Badel J.L., Charkowski, A. O., Deng, W. L., Fouts, D. E., Ramos, A. R., Rehm, A. H., Anderson, D. M., Schneewind, O., van Dijk, K. Pseudomonas syringae Hrp type III secretion system and effector proteins. Proceedings of the National Academy of Science USA 2000.v. 97(16).p.8770-8777.

Licking, E., Gorski, L., Kaiser, D. A common step for changing cell shape in fruiting body and starvation-independent sporulation of Myxococcus xanthus. Journal of Bacteriology; v. 182. p.3553-3558.

Gorski, L., Gornewold, T., Kaiser, D. A sigma-54 activator protein necessary for spore differentiation within the fruiting body of Myxococcus xanthus. Journal of Bacteriology. 2000. v.182. p.2438-2444.

Cooley, M. B., Pathirana, S., Wu, H. J., Kachroo, P., Klessig, D. F. Members of the Arabidopsis HRT/RPP8 family of resistance genes confer resistance to both viral and oomycete pathogens. Plant Cell. 2000. v.12. p.663-676.

Miller, W.G., Bates, A.H., T, H.S., Brandl, M.T., Wachtel, M.R., and Mandrell, R.E. 2000. Detection of *Campylobacter jejuni* cells transformed with new *gfp*, *yfp* and *cfp* marker plasmids on surfaces and in Caco-2 cells Applied and Environmental Microbioogy. V.66 (12). In press.

PROCEEDINGS\ABSTRACTS:

Brandl, M.T., and Mandrell, R.E. Ecological studies of *Salmonella* serotype Thompson on cilantro plants support its role in recent epidemics. Ann. Meeting of American Society for Microbiology. Los Angeles, CA. 2000.

Brandl, M.T., and Mandrell, R.E. 2000. Use of confocal microscopy and the green fluorescent protein in ecological studies on *Salmonella* on plant surfaces. Scanning 2000. 22p. 83.

Harden, L.A., Lieberman, A., Mandrell, R., and Haddon, W.F. A spreadsheet approach to bacterial identification based on MALDI-TOF spectra of whole cells. American Society Mass Spectrometry, Long Beach, CA. 2000.

Mandrell, R.E., Harden, L., Horn, S.T., Haddon, W.F., and Miller, W.G. Analysis of *E. coli* environmental and diarrheal isolates by MALDI-TOF mass spectrometry: Identification of potential biomarkers ions and a mutation in a gene encoding a biomarker ion. Ann. Meeting of American Society for Microbiology. Los Angeles, CA. 2000.

Wood, D.F., Mandrell, R., Bates, A.H., and Yu, P.C. 2000. Immunolocalization of surface antigens on *Campylobacter jejuni* using FESEM and a backscatter electron detector Scanning 2000. 22p. 79-80.

7.26

CRIS Title: Advanced Technologies for Reduction of Microorganisms and Particulate Matter in Food Processing
CRIS: 5325-42000-024
Scientists: Tsai L-S, Hernlem B, Huxsoll C, Robertson G
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Summary Project Aims:
The reduction of microbial populations is vital to the safety, shelf life and quality of food products. The overall goals of this research are to develop advanced engineering and chemical methods for controlling microbial populations in foods by improving the direct disinfection of foods and reducing the opportunities for cross-contamination. The technologies further seek to provide disinfection effectiveness while meeting industry needs for cost efficiency of chemicals, equipment, water, and water disposal. Specifically, we seek the following: 1) develop efficient technology for low cost disinfection application of chlorine dioxide in gas phase while eliminating liquid-mediated cross contamination; 2) improve technologies for liquid phase disinfection using a variety of disinfectants to achieve simultaneous removal by flotation of microbe-containing contaminants and disinfection of food and fluids by in situ electrolytic generation of chemical disinfectants; and 3) develop monitoring and analytical tools that identify all chemical disinfecting species to improve the efficiency of chemical disinfection technology and understand the disinfectant's action and fate in processing environments.

Summary Accomplishments During Entire Project:
Since the inception of this project, we have designed and constructed a novel electroflotation apparatus with non-consumable electrodes to simultaneously disinfect and remove floatable contaminants from food processing water. This apparatus has been used with poultry chiller water, the removal of dissolved protein from model solutions, and the controlled generation of chlorine and gas in various salt solutions under various operating conditions. We have also developed a titration method using a modified pH meter for determination of chlorine species in water and demonstrated its superiority over potentiometric methods. We have also developed an improved monitoring device for the quantification of chlorine dioxide in the presence of chlorine or other chlorine species. Accomplishments in precedent projects include: development of a continuous filtration methodology for reuse of brines, which has been applied at the plant level; establishment of the efficacy of chlorine dioxide in the treatment of poultry chiller water; and establishment of the efficacy of the use of gaseous chlorine dioxide in the treatment of stored potatoes. The gas-phase disinfection of potatoes has led to EPA approval for large-scale testing in Idaho and Washington. This testing is in its third season. One company is working to petition for permanent use status.

Summary 2000 Accomplishments:
The production of safe minimally-processed foods and disease-free seeds could be accomplished by the application of gaseous chlorine dioxide, but the effectiveness of chlorine dioxide gas in these applications has not been tested. ARS researchers at the Western Regional Research Center used chlorine dioxide gas to disinfect a variety of substrates including alfalfa seeds and an inoculated membrane food model. Chlorine dioxide reduced inoculated *E. coli* and naturally occurring flora but also caused some lightening of the substrate surfaces. The gas-phase application of chlorine

dioxide shows significant promise for general use in the fruits and vegetable industry as well as the seed production industry. A key to the production of safe poultry products is the chemical disinfection of the fluids used in the final chilling of the carcass prior to packaging and shipment. ARS researchers at the Western Regional Research Center have developed a prototype electroflotation device that electrolytically generates gas and disinfecting species from salts in the fluids while physically entrapping and removing insoluble and soluble solids in a foam. The team used the electroflotation device to successfully disinfect and clean poultry chiller water obtained from processing plants. With evaluation at a larger scale, this method will provide a very useful industrial method for extending the lifetime of food process fluids and ensure their safety.

Projected Research Accomplishments During Next 3 Years:
Proceed with evaluation of improved electroflotation device. Expand application to other areas such as seafood and produce processing. Continue research on improved sensor and quantification technologies for disinfectants. Establish optimum conditions for gaseous chlorine dioxide treatment of produce that reduces microorganisms without compromising quality. Identify controlling factors in the electroflotation separation and disinfection, and scale-up elecroflotation and gaseous chlorine dioxide disinfection on pathogens, such as *Salmonella* and *E. coli* 0157 H7. Initiate pilot scale electroflotation investigations. Assist CRADA partner to initiate pilot trials and petition for approval with regulatory agencies.

Technology Transfer:
A CRADA has been initiated to evaluate the use of chlorine dioxide in gas-phase disinfection of fruits and vegetables. Expansion of the scope of this CRADA is under negotiation.

PUBLICATIONS:

Hernlem, B.J. and Tsai, L.S., 2000. Chlorine generation and disinfection by electroflotation. J. Food Science, 65(4): 834-837.

Hernlem, B.J.and Tsai, L.S. 2000. Amperometric titration of chlorine with a modified pH meter/tritrator. Accepted by J. American Water Work Association.

Tsai, L.S., Hernlem, B.J. and Huxsoll, C.C. 2000. Disinfection and solids removal of poultry chiller water. Submitted to J. Food Science.

PROCEEDINGS/ABSTRACTS:

Hernlem, B.J., Tsai, L.S., Huxsoll, C.C. and Robertson, G. Combined electroflotation and disinfection in food processing. IFT, Dallas, TX. 2000. Paper #74-12.

CRIS Title: Improve Microbiological Safety & Shelf-Life of Food by Treatment with Ionizing Radiation
CRIS: 1935-42000-033
Scientists: Thayer DW, Fan X, Niemira BA, Rajkowski KT, Sommers CH.
Location: Food Safety Research Unit, ERRC, Wyndmoor, PA
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Summary Project Aims:
The purpose of this research is to improve the microbiological safety and shelf-life of food by treating it with ionizing radiation alone or in combination with other food processing technologies. The project addresses problems that have resulted in several recent outbreaks of foodborne disease and recalls due to microbiological contamination of ground beef, poultry, processed meats, fruits, fruit juices, and vegetables. The effects of pasteurization doses of ionizing radiation (gamma, electron, or X-ray) will be determined on both foodborne pathogens and spoilage organisms and the quality attributes of the associated food product (e.g., ground meat or poultry, processed meats, fruit juices, food sprouts or the seeds from which the sprouts are grown). Of particular interest are the following foodborne pathogens: Escherichia coli O157:H7, Listeria monocytogenes, Salmonella spp., Staphylococcus aureus, and Yersinia enterocolitica. Since food processing technologies with different mechanisms of action such as heating and irradiation have produced greater pathogen inactivation than would be predicted from the sum of the individual processes, complementary and synergistic technologies will be investigated. The effects of ionizing irradiation with other processing or storage technologies and with appropriate food additives on the desired quality attributes of each food will be determined.

Summary Accomplishments During Entire Project:
Our previous research has demonstrated that both intrinsic and extrinsic factors may influence the inactivation of foodborne pathogens. Some of these factors are the atmosphere, pH, and temperature before, during, and after irradiation; previous adaptation of the pathogen to stress; the genetic background and repair mechanisms of the particular organism; the chemistry and physical characteristics of the particular food stuff upon which the contaminants are located; and competition between surviving pathogens and indigenous microflora under stressed conditions, such as modified atmosphere packaging.

Summary 2000 Accomplishments:
Several outbreaks of disease and massive recalls due to contamination of processed meats with Listeria monocytogenes occurred recently; this study was initiated at the Eastern Regional Research Center to investigate the possibility of inactivating this pathogen on frankfurters. Contamination of frankfurters apparently occurs after the heat processing step and unfortunately L. monocytogenes can multiply at refrigeration temperatures. Several types of commercially available frankfurters were surface-inoculated with L. monocytogenes and the radiation dose required for inactivation of the pathogen were determined. A 99.999% inactivation of this pathogen was achieved with a radiation dose of 3.6 kGy; however, differences in radiation sensitivity were discovered that depended on the product formulation. These results meet the goal established by the Food and Drug Administration

for this foodborne pathogen. Yersinia enterocolitica is a foodborne pathogen that is a frequent contaminant of pork products, that is capable of growth at refrigeration temperatures, and whose virulence is dependent on the presence of a large plasmid. The effects of ionizing radiation on the stability of Y. enterocolitica's virulence plasmid was determined. The results of these studies indicate that not only is ionizing irradiation a good method for the inactivation of Y. enterocolitica but, in addition, the number of cells that have lost their virulence plasmid increases ten-fold after treatment with a sub-lethal dose of ionizing radiation. These results indicate that if a few cells of the pathogen should survive treatment with ionization that they would be less virulent. Few commercial food irradiation sources have the means to control temperature during processing of the food; however, the temperature during irradiation processing is known to significantly affect the efficacy of inactivation of pathogens as well as the sensorial properties. The radiation resistance of both E. coli O157:H7 and Staphylococcus aureus were significantly higher in hard-frozen (-20C or lower) than in refrigerated non-frozen ground beef. D-values for the inactivation of these pathogens were determined from -76C to +20C to provide guidance for industry and regulatory agencies. Concerns have been expressed that the inactivation of the indigenous normal microflora by irradiation might allow a pathogen such as L. monocytogenes to multiply much more rapidly. Ground turkey was irradiated, then inoculated with L. monocytogenes, then placed in air-permeable packaging or in packages with 30 or 53% carbon dioxide, and then stored for up to 28 days at 7C. L. monocytogenes did not multiply faster during storage on irradiated than on non-irradiated ground turkey and there was a concentration-dependent inhibition of its multiplication by carbon dioxide. Irradiating ground turkey did not decrease its safety when it was contaminated following processing with L. monocytogenes.

Several outbreaks of salmonellosis have been linked to the ingestion of contaminated orange juice; this study was conducted to determine if treatment with ionizing radiation can provide an alternative to thermal pasteurization for the inactivation of this pathogen. The effects of ionizing radiation dose, turbidity (percent solids), antioxidant activity, and the effect of irradiation in the frozen state on the inactivation of outbreak strains of Salmonella added to orange juice were determined. A 99.999% inactivation of the most resistant strain of Salmonella was achieved at an absorbed dose less than that currently set for non-frozen meat; this will meet the goal set by the FDA for its inactivation.

The use of ionizing irradiation is an effective means to eliminate insects and enhance food safety of many fruits including the apple; however, the impact of irradiation on the production of aroma compounds was unknown. Aroma is an important quality attribute of fresh apple fruit. 'Gala' apple fruit were irradiated to doses of 0 to 1.32 kGy and the production of volatile compounds measured on the day of irradiation and at 1, 3, 7, 14, and 21 days after irradiation. Irradiation at doses sufficient to meet quarantine requirements only temporarily reduced production of volatile compounds of apple fruit. These results indicate that apples can be irradiated without loss of aroma, and the information benefits the industry and the consumer. There have been several recent outbreaks of salmonellosis and infections with Escherichia coli O157:H7 linked to the consumption of raw sprouts. Alfalfa, radish, and broccoli sprouts were inoculated with E. coli O157:H7 or Salmonella and irradiated to doses up to 2.7 kGy. A dose of 2.7 kGy or 1.7 kGy increased the shelf-life and inactivated 99.999% of Salmonella or E. coli O157:H7, respectively, on the sprouts.

Treatment with ionizing radiation at these doses would achieve the goal established by the FDA for treatment of these products. Outbreaks of salmonellosis and severe diarrhea have occurred because of the ingestion of alfalfa sprouts grown from contaminated seeds. Alfalfa seeds from two sources were artificially inoculated with either E. coli O157:H7 or Salmonella and their resistance to gamma radiation was determined. The radiation doses required to inactivate 90% of E. coli O157:H7 or Salmonella on alfalfa seeds were 0.60 or 0.96 kGy, respectively. This process in combination with treatment of the seeds with calcium hypochlorite achieved the 99.999% inactivation of the pathogens desired by the FDA. If sprout seeds are irradiated to inactivate foodborne pathogens it may be necessary to have methods that will allow regulatory agencies to ensure that the seeds have indeed been irradiated. Alfalfa seeds were irradiated and stored for various periods of time before assay. Irradiated seeds were identified by measurement of the hydrocarbons 8-heptadecene and 1, 7-hexadecadiene produced by radiolysis of oleic acid by solid phase micro extraction and identification with GC-MS or by a micro column technique and gas chromatography. A third method was also evaluated in which the free radical signal generated in the very dry seeds was found to be measurable for periods of up to six months from the time of irradiation. All three methods are relatively simple and can detect seeds that have received radiation doses of 1 kGy and will meet the needs of regulatory agencies.

Projected Research Accomplishments During Next 3 Years:
Publication of initial studies on the inactivation of L. monocytogenes on frankfurters, Salmonella spp. and E. coli O157:H7 on alfalfa seeds and sprouts, Salmonella spp. in orange juice, competition of L. monocytogenes with the indigenous microflora on irradiated ground turkey under a modified atmosphere, chemical and spectrometric (EPR) detection of irradiated sprout seeds, effect of irradiation of alfalfa seeds on the yield of sprouts, and effect of irradiation on quality attributes of apples. Completion of the modeling of the effects of irradiation processing temperature on the survival of E. coli O157:H7, Salmonella spp., and S. aureus. Completion of studies of the inactivation of foodborne pathogens on other types of seeds used for the production of food sprouts. Completion of studies of the effects of the composition of the batter used to make frankfurters on the survival of pathogens during irradiation. Initiation of studies on the inactivation of salmonellae in various juices by irradiation and on the quality of the irradiated juice. Initiation of studies of the potential for the use of irradiation processing to inactivate foodborne pathogens on fresh fruit and produce. Completion of studies on the detection of irradiated seed. Completion of studies on the sensory attributes and qualities of irradiated sprouts. Completion of studies on the sensory and quality attributes of irradiated orange juice. Completion of studies on the effects of irradiation processing on the plasmid mediated pathogenicity of Y. enterocolitica. Completion of studies of the effects of irradiation processing with MAP on the extension of shelf-life for ground meat. Completion of studies exploring the possibilities for irradiation inactivation of foodborne pathogens on food sprouts, seed used for the growth of food sprouts, juice, selected fruits, processed meats, and ground meat and poultry packaged using MAP. As in all research projects, additional areas requiring research will be identified.

8.4

Technology Transfer:
Presentations were made to representatives of the sprout, meat, poultry, meat, and nuclear industries. Published data and references have been provided to several firms and to regulatory agencies and the U.S. General Accounting Office.

PUBLICATIONS:

Thayer, D.W., Rajkowski, K.T. Developments in irradiation of fresh fruits and vegetables. Food Technology. 1999. v.53(11). p.62-65.

Engeljohn, D.L., Bell, B.P., Thayer, D.W., Harland, B. Effects of gamma irradiated, cooked soy-, beef-, and soy/meat-based diets in rats. Ecology of Food and Nutrition. 1999. v.38. p.469-490.

Rajkowski, K.T., Thayer, D.W. Reduction of Salmonella spp. and strains of Escherichia coli O157:H7 by gamma radiation of inoculated sprouts. J. Food Protection. 2000. v.63 (7). p.871-875.

Sommers, C.H., Thayer, D.W.. Survival of surface inoculated Listeria monocytogenes on commercially available frankfurters following gamma irradiation. J. Food Safety. 2000. v.20(6). p.127-137.

Thayer, D.W. Sources of variation and uncertainty in the estimation of radiation D-10 values for foodborne pathogens. 2000. ORACBA News. v.5(4). p.1-5.

Fan., X., Argenta, L., Mattheis, J.P. Inhibition of ethylene action by 1-methylcyclopropene prolongs storage life of apricots. 2000. Postharvest Biology and Technology. 20:135-142.

Fan, X., Mattheis, J.P. Yellowing of broccoli in storage is reduced by 1-methylcyclopropene. 2000. HortScience. 35:885-887.

Argenta, L., Fan, X., Mattheis, J. Delaying establishment of controlled atmosphere or CO_2 exposure prevents 'Fuji' apple CO_2 injury without excessive fruit quality loss. 2000. Postharvest Biology and Technology. 20, 221-229.

PROCEEDINGS/ABSTRACTS:

Fett, W.F., Rajkowski, K.T., Thayer, D.W. Chemical, physical and biological methods for controlling human pathogens on sprouts. UJNR Protein Resources Panel, 28th Annual Meeting, Tsukuba, Ibaraki, Japan. 1999. p.84-89. Proceeding.

Thayer, D.W., Rajkowski, K.T., Fett, W.F., Boyd, G. Irradiation of food sprouts and seeds. Food Irradiation 2000. 2000, June. Presentation No. 3. p.1-14. Proceeding.

Thayer, D.W. Irradiation of poultry meat. Proc. 35th National Meeting on Poultry Health and Processing, 2000. Oct. Ocean City, MD. pg87-93.

Fan, X., Thayer, D.W., Niemira, B.A., Mattheis, J.P. Quality of fruit and fruit juice irradiated for the control of food-borne pathogens. UJNR Protein Panel Meeting. Honolulu, Hawaii. 2000. Nov.

Argenta, L.C., Mattheis, J.P., Fan, X. Storage quality and CO_2 injury of Fuji apple fruit. Proceeding of 3rd National Meeting on the Culture of Temperate Fruits, Fraiburgo, SC, Brazil, 2000. p.170-172.

Argenta, L.C., Mattheis, J.P., Fan, X. Impact of 1-MCP (1-methylcyclopropene) on the keeping quality of apples and pears. Proceeding of 3rd National Meeting on the Culture of Temperate Fruits, Fraiburgo, SC. Brazil, 2000. p.129-133.

Sommers, C.H. and Thayer, D.W. Control of Listeria monocytogenes by irradiation. National Food Processors Association. Oct 1999. Chicago, IL. Abstract.

Thayer, D.W. Ionizing irradiation. Application of non-thermal pasteurization technologies to foods. Eastern Food Science Conference XI, Oct. 10-12, 1999. Hershey, PA. Abstract.

Thayer, D.W. Irradiation as a final intervention step. Trends, Technology and Issues Impacting the Future of Food and Beverage Safety. Ecolab Editor's Conference. Sep. 9-10, 1999. St. Paul, Minnesota. Abstract.

Thayer, D.W. Inactivation of foodborne pathogens by irradiation. American Nuclear Society. June 5 2000, San Diego, CA. Abstract.

Thayer, D.W. The microbiological ecology of irradiated meat products. 2000 Institute of Food Technologists Annual Meeting. Jun 2000. Dallas, TX. Abstract. 6-3 p. 8.

Thayer, D.W. Effect of irradiation temperature on inactivation of Escherichia coli O157:H7 and Staphylococcus aureus. Annual Meeting IAFP Atlanta, GA. Aug. 6-9, 2000. Abstract. T26 p. 95.

Thayer, D.W., Boyd, G., Rajkowski, K.T., Fett, W.F., Li, N., Gates, R. Elimination of E. coli O157:H7 and control of Salmonella on sprouts and alfalfa seed by gamma irradiation and sanitation. Annual Meeting IAFP Atlanta, GA. Aug. 6-9, 2000. Abstract. S02, p. 108.

Rajkowski, K.T. Gamma Irradiation. Approaches to control pathogens in the next millennium. Annual Meeting IAFP Atlanta, GA. Aug. 6-9, 2000. Abstract. S05, p. 109.

Sommers, C.H. Loss of crystal violet binding activity in Yersinia enterocolitica following gamma irradiation. Annual Meeting IAFP Atlanta, GA. Aug. 6-9, 2000. Abstract. P030, p. 47.

Thayer, D.W., Boyd, G. Effect of irradiation temperature on inactivation of E. coli O157:H7 and Staphylococcus aureus. Dosimetry for Radiation Processing. 2000. San Diego, CA. Abstract.

Mattheis, J., Argenta, L., Fan, X. Multiple applications extend duration of 1-methylcyclopropene-induced responses of apple and pear fruit. 2000. Hortiscience 35(3):408. Abstract.

Fan, X., D. Buchanan, L. Argenta and J. Mattheis. Ethylene mediates responses of 'Gala' apple to ionizing radiation. 2000. HortiScience 35(3): 408. Abstract.

CRIS Title: Assurance of Microbiological Safety of Thermally Processed Foods.
CRIS: 1935-42000-028
Scientists: Juneja VK, Novak J, Huang L
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Summary Project Aims:
The use of heat to inactivate foodborne pathogens is a critical control point and the most common means of assuring the microbiological safety of cooked foods. A key to optimization of the heating step is defining the target pathogen's heat resistance. Accordingly, the overall goal of the proposed research is to identify the impact of emerging technologies, including processes, packaging methods, or additives, on heat resistance and survival potential of a variety of foodborne pathogens and establish criteria for determining the microbiological safety of milder preservation technologies. We hypothesize that the heat resistance of pathogens can be lowered by incorporating multiple barriers in foods. Thus, we propose to conduct multiple factorial experiments to define and quantify the effects and interactions of intrinsic and extrinsic factors in food in lowering the heat resistance of foodborne pathogens; and develop "enhanced" predictive thermal death, cooling deviation, and integrated kinetics models for inactivation and growth as applied to cooked foods. These predictive equations for the microbiological safety of cooked foods will be incorporated into the USDA pathogen modeling computer program available on the ERRC website. Additionally, we plan to conduct fundamental research on cellular and molecular mechanisms of heat resistance of spores and vegetative cells of pathogens.

Summary Accomplishments During Entire Project:
The safety of minimally processed foods depends on ensuring that any potentially hazardous microorganisms likely to be present in the food are inactivated during heating. Accordingly, we assessed the effects and interactions of temperature (55 - 62.5C), pH (4.0 - 8.0), sodium chloride (0 - 6%) and sodium pyrophosphate (0 - 0.3%) on the inactivation of *Escherichia coli* O157:H7 and *L. monocytogenes*. The multiple regression equations (thermal death predictive models) for these pathogens, which can predict D-values for any combinations of temperature (55 - 62.5C), pH, sodium chloride and sodium pyrophosphate that are within the range of those tested, were developed. Using the inactivation kinetics or predictive models for *E. coli* O157:H7 and *L. monocytogenes*, food processors can design thermal processes for the production of a safe food with extended shelf life without adverse affects on the quality of the product. *Escherichia coli* O157:H7 outbreaks have been primarily associated with undercooked ground beef. We collaborated with the Hospitality Institute of Technology and Management, St. Paul, MN, to quantified the inactivation of *E. coli* O157:H7 and spoilage aerobic microflora in ground beef patties cooked in a skillet. It was demonstrated that the destruction of *E. coli* O157:H7 is predictable if the hamburger is cooked in a way so that the center of the hamburger is measured as the cold spot. The data were used by USDA-FSIS and Health Canada in the development of quantitative risk assessment models; FSIS used the findings to develop set of time/temperature standards to validate the safety of a hamburger cooking process in the retail food operation.

Stressful conditions including sub-lethal temperatures may render foodborne pathogens more thermotolerant. Therefore, the time and temperature needed to inactivate *L. monocytogenes* and *E. coli* O157:H7 in stressed and undercooked beef, respectively, was defined and the mechanism for induced thermotolerance was identified. It was established that (a) an altered fatty acid composition of *L. monocytogenes* could account for changes in heat resistance of *L. monocytogenes* strain Scott A and (b) *E. coli* O157:H7 bacteria that only get a sub-lethal dose of heat can become more heat resistant than bacteria that are not exposed to sub-lethal heat. The findings helped the food industry and regulatory agencies to establish thermal processing guidelines so that stresses do not render bacteria better able to survive thermal processing procedures that normally would be considered adequate.

Improper storage and/or inadequate cooling practices in retail food operations have been cited as cause of food poisoning for 97% of *C. perfringens* and 34% of *C. botulinum* outbreaks. we acquired quantitative data on the growth of *C. perfringens* and *C. botulinum* over the entire growth temperature range which foods must pass through during cooling after cooking. We developed models to predict the relative growth of the pathogens from spores at temperatures relevant to the cooling of cooked products. The growth data were used by the retail food industry to establish guidelines for safe cooling rate of cooked beef and served as the scientific basis for the FSIS regulation on performance standard for the production of cooked meat and poultry products and compliance guidelines for cooling heat-treated products (CFR 64(3) :732-749, Jan. 6,1999, Docket No. 95-033F & Appendix B; Proposed rule, CFR 61(86):19564, 1996, Docket No. 95-033P; and Technical paper, FSIS, Dec. 31, 1998).

Summary 2000 Accomplishments:
Inadequate cooling of foods in retail food operations may allow *C. perfringens* to grow to potentially hazardous infective dose levels. We established the safe cooling rate for cured beef, pork, chicken and uncured chicken by defining the time and temperature needed to ensure safety in relation to control of *C. perfringens* during cooling. A predictive model was developed to predict their growth from spores at temperatures applicable to the cooling of cooked meat. The growth data on the safe cooling rate of cured beef, pork, chicken and uncured chicken will enable regulatory agencies and the food industry to evaluate the safety of cooked products after cooling and thus, with the disposition of products subject to cooling deviations. It is important to understand the basis for pathogen survival to heat treatments used to ensure food safety. Molecular analyses of *C. perfringens* cells following sublethal heating were compared in order to characterize the physiological basis and potential for increased pathogen heat resistance. Various molecules were enhanced in cells as a result of sublethal heating such as molecular chaperones, ribosomal proteins, and small acid soluble proteins, however, all heat-induced effects were lost upon incubation at 4 C for 24 h. These findings will enable the food industry and regulatory agencies to establish safeguards in food handling procedures following processing to ensure that the heat resistance properties of pathogens are not enhanced by these cellular survival mechanisms.

A method was necessary to ensure detection of heat-injured *L. monocytogenes* cells that are capable of repair and survival under various conditions. Reverse transcription PCR was used to detect *L. monocytogenes* at levels of 3 CFU/g of ground beef. It was found that over 60% of the cells were injured after 1 min at 60 C, but 3,000,000 CFU/g of *L. monocytogenes* heat-injured survivors could not be detected as a result of rapid mRNA degradation. These findings will be used by regulatory agencies involved in monitoring safety levels of this pathogen in cooked foods.

Projected Research Accomplishments During Next 3 Years:
The effects and interactions of multiple food formulation variables (NaCl, pyrophosphates, etc.) on thermal inactivation of a cocktail of *Salmonella* spp. and. *L. monocytogenes* in beef with high fat levels will be assessed and subsequently, predictive inactivation kinetics models will be developed. Also, investigations of the molecules responsible for increased heat resistance of *C. perfringens* spores will be continued. Simulating the cooling of cooked meat, the impact of cooling rates for beef and poultry supplemented with various acidulants on the growth of *C. perfringens* from spores will be assessed and the time and temperature needed to ensure safety in relation to control of *C. perfringens* will be defined. Subsequently, predictive models for the fate of *C. perfringens* through out the cooling temperature range of meat products will be developed. Also, molecules playing significant roles in *C. perfringens* spore heat resistance will be isolated and attempts will be made to specifically inactivate their activity or role. Effect of adaptation to environmental stresses on pathogen's (*Salmonella* spp., *L. monocytogenes*, *E. coli* O157:H7, and *C. perfringens*) subsequent heat resistance will be quantified. Additionally, growth or integrated lethality of pathogens during linearly rising (slow and rapid) temperatures of *sous-vide* cooked beef will be determined. Cooking parameters and food formulations will be defined to specifically inactivate molecules necessary for heat-resistant properties in *C. perfringens* spores.

Technology Transfer:
The time and temperature needed to inactivate foodborne pathogens in meat and the safe cooling rates for cooked meats were sent to FSIS and the food industry. The predictive equations were written into an easy-to use computer program and were incorporated in the USDA Pathogen Modeling Program.

PUBLICATIONS:

Juneja, V. K., Whiting, R. C., Marks, H. M., Snyder, O. P. Predictive model for growth of *Clostridium perfringens* at temperatures applicable to cooling of cooked meat. 1999. Food Microbiol. v.16. p.335-349.

Juneja, V. K., Marks, H. M. Proteolytic *Clostridium botulinum* growth at 12 to 48°C simulating the cooling of cooked meat: development of a predictive model. 1999. Food Microbiol. v.16. p.583-592.

Juneja, V. K., Marmer, B. S. Lethality of heat to *Escherichia coli* O157:H7: D- and z-values determinations in turkey, lamb and pork. 1999. Food Res. Internat. v.32. p.23-28.

Juneja, V. K., Eblen, B. S. Predictive thermal inactivation model for *Listeria monocytogenes* with temperature, pH, NaCl and sodium pyrophosphate as controlling factors. 1999. J. Food Protect. v.62(9). p.986-993.

Juneja, V. K., Eblen, B. S. Heat inactivation of *Salmonella typhimurium* DT104 in beef as affected by fat content. 2000. Lett. Appl. Microbiol. v.30. p.461-467.

CRIS Title: Development of Intervention Processes to Enhance the Microbiological Safety of Heat Sensitive Foods
CRIS: 1935-41420-004
Scientists: Kozempel MF, Geveke DJ, Craig JC, Goldberg N, McAloon AJ
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Summary Project Aims:
Pathogenic bacteria exist on the surface of sound meat, such as fresh chicken, occur on ready-to-eat meats, and in liquid foods such as liquid egg and juices. Typical bacteria are E. coli, Salmonella, Campylobacter, and Listeria. These bacteria compromise the safety of our food supply. The objective of the research is to reduce microbial contamination on the surface of solid foods without significant loss in quality and to develop new low temperature pasteurization technology for thermally sensitive liquid foods such as egg. For solid foods, we have been developing an innocuous process which kills bacteria on the surface of meat through the combination of vacuum and steam. First, vacuum is applied to remove air and water on the surface. This permits steam to make instantaneous and direct contact with bacteria on the surface. This is followed by another exposure to vacuum which removes the condensed steam and cools the surface preventing cooking the surface. For liquid foods, we developed a continuous microwave process to test the controversial theory of nonthermal pasteurization with electromagnetic energy. The patented process uses a unique system that removes the microwave energy as soon as it passes through the flowing liquid.

Summary Accomplishments During Entire Project:
The Surface Pasteurization process was conceived, designed, fabricated, and developed for chicken. Proved the concept in a small prototype unit. Based on this success, developed a pilot plant unit. In cooperation with industry, identified a difficulty in treating the visceral cavity of chicken carcasses. Developed a modification to treat the cavity and determined optimum process parameters. The process treatment time is equal to the typical line speed of most chicken processing plants. This research has laid the foundation for planned field tests and eventually for commercial adoption to assure safer food products such as chicken.

Nonthermal or low temperature pasteurization of liquids, such as egg, with microwave or radio-frequency energy is controversial. We developed a continuous microwave process to test the theory. The unique, patented process removes the microwave energy as soon as it passes through the flowing liquid. It separates the effects of thermal energy from nonthermal energy. Microwave energy alone did not destroy microorganisms at low temperatures. Made similar modifications to a radio frequency (RF) oven to repeat the previous microwave experiments at a lower electromagnetic frequency. If nonthermal pasteurization becomes a reality, it would revolutionize liquid egg processing. Even with an increase in processing cost egg processors should rapidly adopt it because it would greatly enhance storage and the physical and functional properties of pasteurized liquid egg as well as improve the safety of liquid egg. It would expand the markets for liquid egg. This is high-risk research with huge potential.

Summary 2000 Accomplishments:
To convince industry to commercialize the process of VSV surface pasteurization of chicken, we must demonstrate its effectiveness on-line. To this end we have designed and fabricated a field processor to run the process at processing plants. The shakedown phase is complete and field tests are commencing. Successful field tests should lead to commercialization and increased food safety for raw chicken.

We agreed to a CRADA to commercialize the VSV surface pasteurization process for the ready-to-eat product industry. The cooperator is designing a commercial packaging unit for hot dogs incorporating the VSV surface pasteurizer as a step in the packaging process. We determined optimum process conditions to achieve 5 log kill of Listeria innocua, which simulates an analog of Listeria monocytogenes. The commercial unit should be available to the industry in about a year and help achieve zero tolerance for L. monocytogenes.

Liquids were exposed to radio frequency (RF) energy to study nonthermal effects on the inactivation of microorganisms. At the frequency and field strength used, neither nonthermal effects nor synergistic effects of RF energy with thermal energy on E. coli K-12, L. innocua, and yeast in apple cider, beer, liquid whole egg, and tomato juice were observed. However, the use of much higher field strengths or lower frequencies holds the potential to inactivate microorganisms.

Projected Research Accomplishments During Next 3 Years:
Perform field tests of the VSV surface pasteurizer on chicken and establish the extent of bacteria kill to be expected on-line. In consultation with the CRADA partner, have a hot dog VSV surface pasteurizer designed and fabricated. Develop a treatment chamber that can be used with the existing RF generator to expose microorganisms to an electric field strength of about 10 kV/cm.Develop a bench scale, batch system to expose liquids to RF energy at a variety of frequencies in the range of 100 kHz to 1 MHZ.

Experiment with the hot dog VSV surface pasteurizer and, in cooperation with the CRADA partner, develop a commercially feasible unit. Perform field tests of the VSV surface pasteurizer on catfish, establish the level of bacteria kill, and the cooperator will determine any organoleptic changes. Develop a bench scale, continuous system to expose liquids to RF energy at a frequency in the range of 100 kHz to 1 MHZ.

Consult in commercial development of the VSV surface pasteurizer. Explore application of the VSV surface pasteurization process to other temperature sensitive foods. Establish RF process parameters such as field strength, frequency, and treatment time that kill bacteria at low temperatures.

Technology Transfer:
The status of both research areas is updated periodically on our web site. Cooperation has been set up with the Poultry Processing and Meat Quality Research Lab, ARS, Athens, Georgia to perform field tests on chicken. One poultry company has requested to observe the tests and several companies have agreed to test the unit at their plants. A CRADA was signed to develop a VSV

surface pasteurizer for ready-to-eat meats beginning with hot dogs. A commercial unit should be available to industry in about two years.Cooperation is continuing with Mississippi State University to reduce microorganisms on catfish. Discussions have been initiated with two other ARS laboratories for possible cooperation in developing the VSV surface pasteurization process for fruits and vegetables. Nonthermal pasteurization of liquids is scientifically controversial. Numerous companies have expressed interest in it pending successful demonstration of the phenomena.

PUBLICATIONS:

Kozempel, M., Goldberg, N., Radewonuk, E.R., Scullen, O.J. Rapid hot dog surface pasteurization using cycles of vacuum and steam to kill Listeria innocua. Journal Food Protection. 2000. v. 63(4). p. 457-461.

Brunkhorst, C., Ciotti, D., Fredd, E., Wilson, J.R., Geveke, D.J., Kozempel, M. Development of process equipment to separate nonthermal and thermal effects of RF energy on microorganisms. Journal Microwave Power Electromagnetic Energy. 2000. v. 35(1). p. 44-50.

Geveke, D.J. "Development of equipment to separate nonthermal and thermal effects of radio frequency energy on microorganisms" at CoFE99 - Sixth Conference on Food Engineering. Dallas, TX. 1999.

PROCEEDINGS/ABSTRACTS:

Geveke, D.J. "The combined effects of RF energy and thermal energy on microorganisms" at American Society of Agricultural Engineers Annual International Meeting. Milwaukee, WI. 2000.

Kozempel, M. Vacuum and steam for surface pasteurization" at A Specialized Food Safety Work Conference 'Reducing Foodborne Illness: Advancing Adoption of New Technologies' sponsored by the Riley Memorial Foundation December 13, 1999.

8.14

CRIS Title: New Technologies to Improve and Assess Food Safety in Muscle Foods
CRIS: 1265-41420-002
Scientists: Nedoluha PC, Berry BW, Solomon MB, Spanier AM.
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Summary Project Aims:
Control of foodborne pathogens and the reduction in the potential health risks to consumers from pathogens in meat products is a most important food safety goal. Recent outbreaks and recalls involving Escherichia coli O157:H7, Listeria monocytogenes, and Salmonella have caused the meat industry to search for new approaches to eliminate pathogen contamination. Traditional postharvest processing technologies, e.g., canning, fermentation and drying, irradiation, hydrostatic pressure processing can not satisfy all of the consumer and industry requirements for minimally processed convenient, affordable and safe food products. The focus of this research is to reduce the incidence of foodborne pathogens in meat products using nonthermal processing technologies. Processed employing hydrodynamic pressure (HDP) technology, plasma pulse sparking technology and other non pyrotechnic methods of generating shock waves are being investigated for the inactivation of microorganisms in meat products. Previous research in this laboratory resulted in the development of consumer messages regarding cooking of hamburgers which stipulated that the only method to insure safety was to use a rapid response meat thermometer and cook to 70 C. However, the potential for misuse and cross contamination with such temperature devices by consumers is possible. Determining the conditions of pathogen contamination with these devices and subsequent development of proper use of temperature monitoring devices for assessing food safety in meat products are required.

Summary Accomplishments During Entire Project:
HDP in a small scale commercial prototype container successfully reduced (4 logs) EHEC ground beef. Normal spoilage microorganisms were reduced (2-3 logs) in ground beef and whole beef and pork muscle. HDP increased the shelf-life of refrigerated ground beef and whole muscle cuts. HDP technology exhibited an additive effect when combined with a second preservation process. These accomplishments provide a basis for further investigations to include determining the action of HDP on bacterial cells. Accomplishments associated with the cooked color problems in beef patties were: (1) cooked beef patty color is an unreliable indicator of internal temperature, (2) high pH muscle (>6.0) can lead to limited myoglobin denaturation during cooking and thus, produce considerable pink color well above 160 F, (3) cooking patties from the thawed state can lead to premature brown color, (4) controlled thawing, however, can be a useful tool in preventing the persistent pink color of high pH patties, (5) considerable temperature variability can exist within patties and between patties cooked under the same conditions, (6) termination of cooking by observing brown color in slits in patties cooked on a gas grill is not synonymous with a safe internal temperature, (7) appearance of brown color in beef patties several minutes after cooking should not be used as a criteria of food safety due to continued myoglobin denaturation with our continued temperature increase in patties, and (8) with thin patties (0.94 cm) increases in temperature post-cooking are often

not sufficient to guarantee food safety if the patties are removed from electric grills between 66.1 and 68.3 C. These accomplishments have resulted in new and revised recommendations to food service operations and consumers regarding cooking of beef patties.

Summary 2000 Accomplishments:
Consumers are demanding high quality muscle foods that are minimally processed, free of additives and residues, healthy, convenient, and affordable. Data suggests that Gram-negative bacteria, specifically the pseudomonads, which are the predominant organisms that cause spoilage of fresh meats, are more susceptible to hydrodynamic pressure processing (HDP). HDP is a newly emerging nonthermal postharvest technology being developed to simultaneously improve meat safety and shelf-life as well as meat tenderness. The decrease in pseudomonads resulting from HDP treatment causes an extension of meat product shelf-life. Results also indicate that when HDP is combined (Hurdle concept) with an additional preservation process, the effective reduction in Gram-negative bacteria is synergistic, thus establishing successful nonthermal improvements in food safety using HDP.

Foodborne illness has been associated with Escherichia coli O157:H7 bacteria and inadequately cooked ground beef patties. This has highlighted the need for a reliable indicator of thorough cooking. Internal temperature measured in the thickest portion of patties removed from the cooking environment frequently is <65 C when using color as the doneness indicator. With thin patties (0.94 cm), post-cooking increases in temperature are often not sufficient to guarantee food safety if patties are removed from electric grills between 66.1 and 68.3 C (patties often visually appear cooked when these temperatures have been reached). With gas grilling (most popular method of cooking beef patties during summer months) determination that cooked patties have reached a food safe endpoint by the appearance of brown color in a slit made in the outer edge of patties presents a sizable food safety risk.

Projected Research Accomplishments During Next 3 Years:
MSRL will develop a standard system for measuring the consistency of the shock waves generated during the HDP process. This includes a standard processing procedure and representative Gram-negative and Gram-positive microorganism which will consistently have a minimum 3 log reduction following treatment with HDP. We propose to examine damage from HDP and other emerging technologies to cell walls and membranes, proteins, and DNA. The effect of HDP on pathogens, EHEC, Listeria monocytogenes, Salmonella Typhimurium, Yersinia enterocolitica, and Campylobacter jejuni will be determined. HDP will be combined with other preservation processes (irradiation, naturally-occurring compounds) to determine effectiveness.

ARS will attempt partnership with the meat industry customers to commercialize the most efficacious hydrodynamic pressure system for enhancing food safety. Reliability and accuracy of temperature indicating devices in beef patties and boneless chicken breasts will be assessed. The influence of cooking procedures, size of product and composition on the accuracy of the indicators will be determined. Assessment of the risk of cross contamination in the use of the devices will be determined and cleaning and sanitation of protocols for temperature indicators will be developed.

Other new technologies for food safety will be either developed or evaluated and possibly combined with hydrodynamic pressure systems. Systems with the most promise will be subjected to in-plant testing and evaluation for full commercialization.

Technology Transfer:
As a result of ARS research and transferred technology, USDA has implemented an extensive program to: (1) encourage the manufacture of inexpensive and accurate temperature indicating devices, (2) assist retailers in disseminating information on safe cooking of hamburgers, and (3) aid consumers in employing food-safe cooking procedures for hamburgers. Workshops for thermometer manufacturers and retailers have held to initiate this program. Several retailers have inaugurated programs for consumers regarding safe cooking of hamburgers using ARS-developed technology. Adoption of this technology by consumers will be difficult as consumers resist using thermometers for cooking meat and surveys indicate <5 percent of U.S. consumers own and use thermometers in cooking meat. As a result of the findings from this ARS research, FSIS-USDA launched in May 2000, and extensive educational program ("Thermy") to expand the knowledge of and use of accurate meat temperature indicating devices. Commercialization of HDP for reducing pathogens and spoilage microorganisms in meat products is still in the research development stage. Research conducted over the next two years will reveal answers that can be used to commercialize HDP and other emerging technologies evaluated.

PUBLICATIONS:

Berry, B.W., Bigner-George, M.E. Factors affecting color properties of beef patties cooked on an outdoor gas grill. 2000. Journal of Muscle Foods. v. 11. p. 213-226.

Bigner-George, M.E., Berry, B.W. Thawing prior to cooking affects sensory, shear force and cooking properties of beef patties. 2000. Journal of Food Science v. 65. p. 2-8.

Williams-Campbell, A., Solomon, M.B. New non-thermal postharvest technology to improve food safety: hydrodynamic pressure processing. 2000. Society of Photo-Optical Instrumentation Engineers #4206-22.

PROCEEDINGS/ABSTRACTS:

Berry, B.W., Bigner-George, M.E. Color properties of beef patties cooked on an outdoor gas grill as influenced by fat content, product handling and internal temperature. Proceedings, 10th Congress of Food Sci. and Technology, Sydney, Australia, October 4, 1999. p. 65.

Campbell, A., Solomon, M.B. Extending the shelf-life of ground beef using hydrodynamic pressure process. 100th Amer. Soc. for Microbiology, May 23, 2000. p. 537. Abstract #P-115.

Solomon, M.B., Williams-Campbell, A. Hydrodynamic shock waves as a food preservation technique. Inst. of Food Technologists, June 10, 2000. p. 10. Abstract #8-5.

Williams-Campbell, A., Solomon, M.B. Hydrodynamic pressure processing for improving the safety of meat products. Annual Thermal Processing Workshop. Crystal City, Virginia, December 14, 1999. Abstract p. 8.

CRIS Title: Stress Responses and Virulence Expression of Bacterial Pathogens in Food Environments
CRIS: 1935-42000-031
Scientists: Fratamico PM, Bhaduri S, Bayles DO, Solow BT
Location: Microbial Food Safety Research Unit, ERRC, Wyndmoor, PA
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Summary Project Aims:
Understanding the mechanisms that bacteria have evolved to withstand stresses found in nature and in food environments is essential for effectively controlling or eliminating bacterial pathogens in foods. Since these processes are not well understood, an elucidation of the physiological and genetic factors that induce increased tolerance of pathogens to stressful environments and improved methods for detecting stressed/injured bacterial pathogens in food are needed. The specific objectives are: (1) to study the effect of stresses on bacterial survival and on cross-protection/cross-sensitization against subsequent stresses; (2) to examine the effects of stress conditions on bacterial virulence and expression of virulence-associated genes; and (3) to develop methods for detection of stressed/injured pathogens. Understanding the molecular aspects of stress responses in food-borne pathogens will potentially reveal new strategies for food preservation, permit the identification of potential targets that might be exploited in reduction or elimination of pathogens, and also permit the design of innovative interventions which prevent stress adaptation, diminish the virulence of pathogens, and ensure destruction of pathogenic bacteria in foods.

Summary Accomplishments During Entire Project:
The contamination of fruit juices by bacterial pathogens is currently of considerable interest and concern. Initial studies showed that a sensitizing cold treatment applied to *Listeria monocytogenes* Scott A prior to thermal inactivation resulted in thermal death times that were lowered by up to 45% compared to controls, and that changes in the state of the ribosomes correlated with this reduction in thermal sensitivity. In similar studies, data suggested that cold shock prior to pasteurization of fruit juices could provide an extra measure of safety and would also allow juice processors to reduce thermal processing requirements for pathogen control while maintaining the fresh qualities of the juice. This information will lead to the development of more effective processes to eliminate bacterial pathogens from ready-to-eat foods.

A method involving a multiplex polymerase chain reaction (PCR) assay was developed to simplify detection and identification of *Escherichia coli* O157:H7 in foods, and to also permit detection of low levels of bacteria subjected to cold stress. Primers for a plasmid-encoded hemolysin gene (hly_{933}), and chromosomal flagella ($fliC_{h7}$; flagellar structural gene of H7 serogroup), Shiga toxins (stx_1, stx_2), and attaching and effacing (*eaeA*) genes were used in a multiplex PCR for co-amplification of the corresponding DNA sequences from *E. coli* O157:H7. Sensitivity of the assay was ≤ 1 CFU/g of food or bovine feces and results could be obtained within 24 h. Similar detection levels were obtained with ground beef samples that underwent enrichment culturing immediately after inoculation and samples that were frozen or refrigerated prior to enrichment. In addition to allowing for rapid detection and identification of low numbers of *E. coli* O157:H7 in foods and other

types of samples, the multiplex PCR can markedly reduce the time required for confirmation of isolated colonies since lengthy biochemical, serological, and toxin testing could potentially be eliminated. This technology can enhance the ability of the food industry and regulatory agencies in testing for *E. coli* O157:H7 in foods.

S. typhimurium DT104 infections may be associated with a higher morbidity and mortality than infections caused by other *Salmonella* phage types; however, further studies are needed to assess whether DT104 strains are more virulent and/or have a greater ability to tolerate stress than non-DT104 *S. typhimurium* strains. Our research revealed that the *S. typhimurium* DT104 strains that were studied did not show an increased ability to invade mammalian cells compared to non-DT104 strains and did not have an increased ability to survive stress. Acid adaptation of the bacteria did not result in increased invasion of mammalian cells, did not result in increased ability to survive in apple cider, and did not provide cross protection against exposure to hydrogen peroxide, acetic acid, or high salt levels. However, acid adaptation did induce increased resistance to a low pH gastric environment. Results of these studies provide the food industry and the scientific community with information regarding the virulence of *S. typhimurium* DT104 and the ability of this organism to tolerate and adapt to stresses encountered in foods and during food processing. Data indicate that measures taken to inactivate or inhibit the growth of *Salmonella* in foods should also be sufficient to inhibit *S. typhimurium* DT104. The mechanism by which *S. typhimurium* DT104 accumulated resistance genes is of interest since these genes interfere with treatment of infections caused by this organism and they might be horizontally transferred to other bacteria, even to unrelated organisms. The arrangement and the location of the integrons and other resistance genes were determined. A chloramphenicol resistance gene (*cmlA*) was homologous to a *cmlA* exporter gene found in *Pseudomonas aeruginosa*. Both type I integrons possess the classical structure, as evidenced by the presence of a 5' integrase gene and a 3' *sulI* gene, separated by an antibiotic resistance gene and a disinfectant resistance gene, *qacE*Δ. In addition, the integron arrangement is shown to be useful for PCR detection of multi-resistant *S. typhimurium* DT104.

Yersinia enterocolitica is an important cause of gastroenteritis in humans, and outbreaks have been associated with consumption of pork products. A simplified and relatively rapid mouse virulence assay, which does not involve iron pretreatment was developed to evaluate the pathogenicity of *Y. enterocolitica* isolated from pork samples. Elimination of the iron pretreatment step avoids iron-induced stress in the mice and reduces the assay time by two days. The new procedure will allow researchers and clinical laboratories to more readily assess the pathogenicity of strains isolated from foods implicated in human illness. Pathogenic *Y. enterocolitica* survived freezing in ground pork and milk; however, the organism could not be recovered from samples inoculated at less than 10 CFU/cm^2 of pork using a swabbing technique, whereas it could be recovered from pork samples inoculated at 0.5 CFU/cm^2 that were not subjected to freezing for prolonged periods.

Summary 2000 Accomplishments:
Campylobacter is the most common cause of bacterial food-borne illness in the United States despite its fastidious growth requirements. A limited understanding of the genetics, physiology, and virulence factors of *Campylobacter* and of its ability to respond to stressful environments is

hindering our capability to effectively detect and control this organism in food. It was determined that *Campylobacter coli* and *Campylobacter jejuni* survive as well on pork as on chicken skins, the optimal temperature for survival is refrigeration temperature, and the presence of oxygen does not influence viability at refrigeration and freezing temperatures. Results from these studies enhance the understanding of *Campylobacter* stress responses and of conditions which permit its survival in foods. Further, to determine the relatedness of *Campylobacter* strains isolated from rectal swabs and different areas in a pork processing plant, the antibiotic resistance and pulsed field gel electrophoresis profiles were determined. Based on the profiles, each of the 60 isolates examined were distinct, suggesting that numerous *C. jejuni* and *C. coli* strains originating from diverse sources were present in the pork processing plant. A PCR-based assay was developed to simultaneously detect the organism and differentiate between *C. jejuni* and *C. coli*, the two major food-borne pathogenic species. This PCR-based assay can be used by the food industry and regulatory agencies to monitor for the presence of *Campylobacter* in foods and other types of samples.

Understanding the processes underlying osmotic adaptation in *L. monocytogenes* is critical in trying to design ways to control this organism in foods with high osmotic pressure. To facilitate an understanding of how *L. monocytogenes* responds to osmotic stress, a *L. monocytogenes* mutant (OSM1) which had a reduced growth rate compared to the parent strain when grown in a medium with high salt concentrations, when grown at 44°C, and when exposed to oxidative stress (exposure to hydrogen peroxide) was isolated and characterized. Sequence analyses of the mutagenized gene in OSM1 demonstrated homology with genes encoding serine proteases such as HtrA (DegP). This *L. monocytogenes* serine protease may be a previously unidentified stress response gene involved in osmotic, temperature, and oxidative stress responses. Thus, a new gene required for *L. monocytogenes* to survive and grow under conditions of refrigeration, elevated osmolarity, and oxidative stress has been identified. This gene will be targeted in an effort to reduce the survivability and infectivity of *L. monocytogenes* in ready-to-eat foods, for example through the use of compounds that interfere with the expression or function of this protease.

The FDA has proposed testing of spent irrigation water during sprout production for the presence of pathogens as a means for determining the safety of individual batches of sprouts. We evaluated an immunochromatographic assay and a polymerase chain reaction (PCR)-based assay for detection of *E. coli* O157:H7 in spent irrigation water from alfalfa sprouts grown from artificially-contaminated seeds and in enrichments of blanched sprouts. Results indicated that blanching may not be effective to completely inactivate all the *E. coli* O157:H7 that may be present in sprouts, that enrichment of spent irrigation water enhanced the ability to detect the organism, and that PCR-based assays are more sensitive than immunologic-based assays. This information is important for the sprout industry and regulatory agencies for developing strategies to control the presence of this pathogen in sprouts.

Poultry and poultry products are important vehicles of transmission of human salmonellosis and improved methods for detection of *Salmonella* in these and other foods are needed. Thus, a standard cultural method, two PCR-based assays, and an immunochromatographic assay were evaluated for detection of *Salmonella* spp. in naturally-contaminated ground chicken and turkey. The PCR-based

assays were more sensitive and reliable than the immunoassay and the standard cultural method. This information is valuable to regulatory agencies and the food industry in their development of protocols for *Salmonella* testing.

To improve the utility of the PCR for analysis of food samples, various methods for sampling of foods and for preparing template DNA were compared for removal of PCR inhibitors in food samples artificially contaminated with *S. typhimurium*, *E. coli* O157:H7, and *L. monocytogenes*. The presence of each pathogen was confirmed by the PCR, and each of the organisms could be detected even when inoculated in the same food sample. Recovery of bacteria from foods by swabbing can reduce the level of inhibitors of the PCR, thus barring the need to perform time-consuming DNA extraction techniques on samples.

Multiple-antibiotic-resistant *S. typhimurium* G8430, phage type U302, has plasmids that are ca. 3.3 kb and 90 kb in size. The 3.3-kb plasmid was sequenced and found to encode for kanamycin and neomycin resistance. The ca. 90-kb plasmid, which encodes gentamycin resistance, has regions that are highly homologous to the pO157 plasmid from *E. coli* O157:H7 and genes for organic solvent tolerance, an anti-restriction protein, and a modification methylase, and several genes conveying antibiotic resistance. Identification of the genes encoded on these plasmids will help to elucidate the virulence of multiple-antibiotic-resistant *S. typhimurium*. In addition, similar studies will help in the understanding of the mechanisms of acquisition of antibiotic resistance genes in this organism, potentially leading to development of strategies that prevent the spread of antibiotic resistance.

Projected Research Accomplishments During Next 3 Years:
During the next three years we will (1) work with the Food Safety and Inspection Service and collaborate with companies including PE-Applied Biosystems, Qualicon, and Advanced Analytical Technologies that market reagents, instruments, and diagnostic kits to develop rapid and reliable methodologies to detect stressed/injured pathogens in foods, in particular, *L. monocytogenes* and enterohemorrhagic *E. coli* (EHEC) and identify genes in EHEC which can be employed in the design of PCR-based assays for specific detection of serotypes in this group; (2) identify genes/proteins needed for *L. monocytogenes* to survive refrigeration conditions in a food matrix and for *Salmonella* to survive under acid and other stress conditions; (3) use the green fluorescent protein or other reporter system such as β-galactosidase or luciferase to monitor expression of virulence- and stress-related genes in foods; (4) examine the effect of stress on growth and survival of EHEC and on Shiga toxin production and prophage induction; (5) using genetically-engineered *E. coli* strains provided by Dr. James Kaper at the University of Maryland, determine how quorum sensing contributes to the growth, survival, and pathogenicity of *Salmonella*, EHEC, and *Campylobacter* in foods; (6) examine transcription of genes involved in curli production controlled by RpoS and determine the function of curli and conditions under which they are expressed; and (7) identify, characterize, and sequence plasmids, and chromosomal regions in *S. typhimurium*, *Campylobacter* spp., and *Y. enterocolitica* which encode for antibiotic resistance genes and genes involved in virulence of these pathogens, and sequence O antigen gene clusters of several EHEC strains to ultimately develop genetic-based assays for specific detection of *E. coli* belonging to this group.

Technology Transfer:
Research on the development of improved pathogen detection methodologies will be transferred to the ARS stakeholders including regulatory agencies and the food industry through discussions and demonstrations and through dissemination in the scientific literature and at scientific meetings. We have established a dialogue and/or collaborative studies with PE-Applied Biosystems and with Qualicon on development of specific, rapid, and reliable methodologies to detect stressed/injured pathogens in foods, fecal, and other samples. We are working with Wampler Foods to examine survival of pathogens on ready-to-eat foods subjected to different processing procedures and on molecular tracking of pathogens in foods. Research results on bacterial stress responses will elucidate the effects of food environments and food processing and storage conditions on growth and survival of pathogenic bacteria in food, ultimately leading to development of strategies to decrease viability of pathogens in foods. The inducible and enhanced vulnerability of *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 to heat in model and food systems shows the potential for this approach to be used by the food industry as a practical and efficacious post-processing intervention strategy. The knowledge gained by the research will provide the tools and facilitate the development and improvement of practical food preservation systems which minimize health risks.

PUBLICATIONS:

Fratamico, P.M. and Crawford, C.G. 1999. *Escherichia coli* O157:H7: Detection by commercial immunomagnetic particle-based assays, p. 654-661. In Encyclopedia of Food Microbiology, R. Robinson, C. Batt, and P. Patel (ed.), Academic Press Ltd, London.

Fratamico, P.M. 2000. Applications of the polymerase chain reaction (PCR) for detection, identification, and typing of food-borne microorganisms, p. 95-115. In Microbial Food Contamination, C. Wilson (ed.), CRC Press, Boca Raton, Florida.

Fratamico, P.M., Bagi, L.K., and Pepe, T. A multiplex PCR for rapid detection and identification of *Escherichia coli* O157:H7 in foods and bovine feces. J. Food Prot. 63:1032-1037.

Bhaduri, S. and Cottrell, B. 2000. Iron pretreatment is not essential for assessment of pathogenicity of *Yersinia enterocolitica* serotype O:3 in mice. J. Rapid Meth. Automat. Microbiol. 8:1-10.

Bhaduri, S. Pathogenic *Yersinia enterocolitica*. In Guide to Foodborne Pathogens, R.G. Labbe and J.S. Garcia-Alvarado, John Wiley and Sons, Inc., New York, NY (in press).

Bayles, D.O., Tunick, M.H., Foglia, T.A., and Miller, A.J. Cold shock and its effect on ribosomes and thermal tolerance in *Listeria monocytogenes*. Appl. Environ. Microbiol. 66:4351-4355.

Miller, A.J., Bayles, D.O., and Eblen, B.S. Cold shock induction of thermal sensitivity in *Listeria monocytogenes*. Appl. Environ. Microbiol. 66:4345-4350.

PROCEEDINGS/ABSTRACTS:

Fratamico, P.M. and Bayles, D.O. 1999. Control of bacterial pathogens in foods: understanding the mechanisms of stress responses and development of methods for detection of pathogens in foods, pp. 107-117. Proceedings of the 28th United States-Japan Cooperative Program in Natural Resources - Protein Resources Panel Meeting, Tsukuba, Japan.

Cloak, O.M. and Fratamico, P.M. 2000. A multiplex PCR for the detection of *Campylobacter jejuni* and *Campylobacter coli* from pork and characterization of isolates from pork by PFGE and antibiotic resistance patterns. Abstract No. P72. Annual Meeting of the American Society for Microbiology, Los Angeles, CA.

Fratamico, P.M. 2000. Invasive ability and tolerance of acid-adapted and non-adapted *Salmonella typhimurium* DT104 to stress conditions. Abstract No. P41. Annual Meeting of the International Association of Food Protection, Atlanta, GA.

Fratamico, P.M. and Bagi, L.K. 2000. Methods for detection of *Escherichia coli* O157:H7 in alfalfa sprouts and the effectiveness of blanching for inactivation of the organism in sprouts. Abstract No. P8. Society for Industrial Microbiology Conference - Food-borne Pathogens 2000: Perspectives and Interventions, Arlington, VA.

Bayles, D.O. and Wonderling, L.D. 2000. A novel Tn917 mutant of *Listeria monocytogenes* is defective in a serine protease and is sensitive to osmotic, temperature, and oxidative stress. Abstract No. K-16. Annual Meeting of the American Society for Microbiology, Los Angeles, CA.

Bhaduri, S. 2000. Effect of freezing on the isolation and survival of plasmid-bearing virulent *Yersinia enterocolitica* in pork. Abstract No. P38. Annual Meeting of the International Association of Food Protection, Atlanta, GA.

Bhaduri, S. 2000. Simple and rapid preparation of PCR samples from food for detection of *Escherichia coli* O157:H7, *Salmonella typhimurium*, and *Listeria monoctyogenes*. Abstract No. P33. Annual Meeting of the American Society for Microbiology, Los Angeles, CA.

Fratamico, P.M., Solow, B.T., and Cloak, O.M. 2000. *Campylobacter*: characterization, detection, and growth and survival mechanisms. 29th United States-Japan Cooperative Program in Natural Resources - Protein Resources Panel Meeting, Honolulu, HI.

CRIS Title: Post-Harvest Predictive Microbiology and Process Risk Assessment
CRIS: 1935-42000-041 (from merge of 1935-42000-029 and1935-42000-032)
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Summary Project Aims:
In an effort to reduce the impact of foodborne disease, microbial risk assessments serve to identify control points in the food production system which ultimately can reduce the impact of foodborne disease. In the course of developing risk assessments, data gaps are identified which decrease the assessment's level of certainty. A common data gap in risk assessments is insufficient knowledge about the predictive behavior of bacterial pathogens in food. Predictive models of the kinetics of bacterial growth and inactivation in model foods are needed to enhance the accuracy of microbial risk assessments. Among the important foodborne bacterial pathogens, certain *Shigella* spp. are important etiological agents of foodborne gastrointestinal illness. Based on CDC reports, the incidence of *Shigella* spp. infections is estimated at 448,000 cases per year, with 20% of the cases due to foodborne transmission. *Shigella* can be transmitted person-to-person, through fecally contaminated water and by infected food handlers. The infective dose for the disease is relatively low, at 10 to 100 organisms. There are indications that the organism is able to survive for prolonged periods of time in a number of foods with a relatively low pH or low water activity. Prior to this research, few systematic studies have reported the survival of *Shigella* spp. under model food conditions. In addition, human pathogenic bacteria of chicken origin cause an unknown number of cases of gastroenteritis each year. Some cases of gastroenteritis from chicken result in death or chronic disability. To protect consumers from human pathogens associated with chicken, the USDA instituted a microbial performance standard-based HACCP meat inspection regulation in 1996. The new regulation evaluates food safety based on *Salmonella* incidence of chicken near the end of processing. The evaluation should consider all relevant factors, such as other hazards (i.e., *Campylobacter*), post-processing events (i.e., cooking), and public health impact, in its assessment of food safety. To facilitate adoption of a public health performance standard- based HACCP meat inspection regulation, we are also developing computer models that consider relevant factors in quantitative assessment of the public health impact of human pathogen infections from chicken.

Summary Accomplishments During Entire Project:
Within the risk assessment community, there is broad recognition that comprehensive predictive models for the most important foodborne pathogens are lacking. Such models must simulate microbial growth, survival and decline in foods which are recognized as relevant vehicles of disease. The overall goal of this project is to develop robust models for *Listeria monocytogenes*, *Shigella flexneri*, *Escherichia coli* O157:H7, *Campylobacter* spp. and *Salmonella.* Although research is in progress to further refine the predictive models, the current 5.1 version of the Pathogen Modeling Program demonstrates the success of this project, by offering predictive models of *L. monocytogenes*, *S. flexneri*, *E. coli* O157:H7, and *Salmonella* under various environmental conditions. In the past year, we refined a model for the survival of *S. flexneri* under non-thermal inactivation conditions. This new information expands the model for the behavior of *S. flexneri*

under aerobic and anaerobic conditions, as a function of temperature, pH sodium chloride and sodium nitrite concentrations. Furthermore, the model has been validated in various foods, and good agreement was obtained when growth kinetics of the bacterium, as predicted by the model (aerobic), were compared with those observed in foods. In addition, development of methods for modeling the public health impact of human pathogen infections from chicken produced by specified farm to table scenarios has been another accomplishment over the life of this project. These modeling methods form the basis of Poultry Food Assess Risk Model (Poultry FARM).

Summary 2000 Accomplishments:
A comprehensive model for the growth, survival and decline of *S. flexneri* in food is lacking. Within this model, non-thermal inactivation data are needed to predict the survival of *S. flexneri* in food vehicles with high salt and/or low pH. Therefore, we conducted studies of the effect of sodium chloride (0.5, 2, 4, 6, 8%) on survival of *S. flexneri* in broth culture as a function of temperature (19, 28, 37 deg C and pH (4, 5, 6). Sodium chloride concentration had a strong effect on the growth of *S. flexneri*. Under conditions that did not promote growth, increasing sodium chloride concentrations led to a decrease in lag time, while the overall survival times were affected to a lesser extent. Specifically, *S. flexneri* grew at all three temperatures in media of pH 6 containing less than or equal to 6% sodium chloride. At 19 and 28 deg C, the bacteria grew in media of pH 5 containing less than or equal to 4% sodium chloride, while at 37 deg C growth was observed in media of pH 5 containing less than or equal to 2% sodium chloride. Growth was not observed in media of pH 4 and populations gradually declined. A two-phase linear model was used to fit the individual survivor curves from which lag times, D-values and times to 4-D (99.99%) inactivation were derived. These results verify that foods with relatively high salt content can support the survival of *S. flexneri*. This research benefits the efforts of epidemiologists and risk assessors who develop interventions to reduce the impact of human shigellosis. This research complements on-going efforts to develop a comprehensive model of *S. flexneri* growth, survival, and decline in food. In this regard, it supports new research to determine the effect of sodium chloride concentration (0.5-8%) in combination with low temperature (4 and 12^{o}C) and low pH (2 and 3) on survival of *S. flexneri*. In addition, studies have begun to determine the effects of various organic acids (acetic, lactic, malic, citric, tartaric) associated with foods on the survival of *S. flexneri* at low pH. The final product will be a non-thermal inactivation model which will be incorporated into the Pathogen Modeling Program, and made available to risk assessors in industry, government and academia via the internet.

Projected Research Accomplishments During Next 3 Years:
Projects 1935-42000-032 and1935-42000-029 have been terminated, and future efforts will be reported through project number 1935-42000-041. Within the next year, a new project plan will be developed that will greatly expand the capabilities and utility of the Pathogen Modeling Program. Specifically, the models for *L. monocytogenes*, *S. flexneri*, *E. coli* O157:H7, *Campylobacter* spp. and *Salmonella* will be validated in relevant food matrices under various packaging and handling conditions. This will include the development of food-specific models when good agreement is not found with the Pathogen Modeling Program. A five-year research plan will be available following development and acceptance of the final project plan. We also plan to improve Poultry FARM with a planned release of version 2.0. We will conduct further research on the growth and survival of

Salmonella in poultry foods. Currently, we are conducting challenge studies with green fluorescent protein (GFP) *Salmonella* inoculated into poultry foods with the goal of establishing GFP *Salmonella* as a tool for modeling behavior of Salmonella in raw poultry foods. If successful, we will develop comprehensive models for growth and survival of Salmonella in raw poultry foods over a broad range of temperatures (8 to 50 deg C). Four strains of GFP Salmonella will be used in these studies. Models will be validated against data not used in their development and against data collected on sterile poultry foods with poultry isolates of Salmonella and parent strains of GFP *Salmonella*. The new models may be added to Poultry FARM and distributed to stakeholders in version 2.0.

Technology Transfer:
The products of this research have been transferred to individuals and groups via version 5.1 of the Pathogen Modeling Program and version 1.0 of Poultry FARM. These software programs are disseminated to interested users through the internet and by mailing diskettes. Predictive models address growth, and thermal, non-thermal, and radiation inactivation of *L. monocytogenes*, *S. flexneri*, *E. coli* O157:H7, *Campylobacter* spp. and *Salmonella*. Our aim is to include the completed non-thermal inactivation model for *S. flexneri* in the Pathogen Model Program within the next two years. Growth and survival data are also available to other scientists when are published in the literature. Poultry FARM may also be upgraded to include new research-based information to assist in estimations of the risk and severity of *Salmonella* and *Campylobacter* infections from chicken products. The extensive data sets that we have developed have been used by several groups of scientists in the USA, Europe, and Australia to develop other types of growth models.

PUBLICATIONS:

Oscar, T. P. 1999. Response surface models for effects of temperature, pH, and previous growth pH on growth kinetics of Salmonella Typhimurium in brain heart infusion broth. J. Food Prot. 62:106-111.

Oscar, T.P. 2000. Response surface models for effects of temperature and previous growth sodium chloride on growth kinetics of *Salmonella Typhimurium* on cooked chicken breast. J. Food Prot. 62:1470-1474.

PROCEEDINGS/ABSTRACTS:

Zaika, L.L. 2000. Effect of salt on survival of *Shigella flexneri* as affected by temperature and pH. 87th Annual Meeting of the International Association for Food Protection. Paper No. P-051.

Zaika, L.L.,Fanelli, J.S. 1999. The effect of temperature on the survival of *Shigella flexneri* at low pH. 86th Annual Meeting of the International Association of Milk, Food and Environmental Sanitarians. Paper No. P-90.

CRIS Title: New Technologies for Decontamination of Fresh Fruits and Vegetables Containing Human Pathogens
CRIS No.: 1935-41420-003
Scientists: Sapers GM, Annous BA, Sites J., Fett WF, Goldberg NM, Hicks, KB
Contact: 215-233-6417 (P); 215-233-6406 (F); gsapers@arserrc.gov

Summary Project Aims:
The microbiological safety of fresh and minimally processed and packaged fruits and vegetables has been questioned as a result of recent outbreaks of foodborne illness associated with unpasteurized juices, sprouts, melons, lettuce, berries and other commodities. The presence and survival of human pathogens in these commodities have been demonstrated. Current methods of washing and sanitizing produce are largely ineffective. Specific knowledge of sources of microbial contamination and critical control points, required for development of HACCP plans, is deficient. We have developed a broad-based research program to identify sources of human pathogen contamination and develop interventions to prevent contamination, remove or inactivate pathogens on fresh and minimally processed produce. This program will provide inputs for HACCP plan development and other produce industry guidelines for improving microbiological safety.

Summary Accomplishments During Entire Project:
Washing with hypochlorite or commercial surfactant formulations, as a means of decontaminating apples inoculated with *E. coli*, was shown to be limited in efficacy, achieving population reductions of only 1-2 logs (90-99%). In contrast, an experimental washing treatment with hydrogen peroxide resulted in 3-log (99.9%) reductions. However, field tests demonstrated the inability of brush washing to decontaminate apples, even with treatments shown to be efficacious in laboratory trials, probably due to binding of bacteria in inaccessible sites, growth of bacteria in skin punctures, and possible infiltration of bacteria into calyx and core tissues. These results demonstrated the need for improved methods to apply promising sanitizing treatments. Specifications were developed for a Biological Safety Level (BSL)-2 pilot plant facility for use in evaluating new washing technology for decontamination of fresh produce containing human pathogens. An agreement between ARS and Penn State University (PSU) was negotiated to facilitate the design, fabrication and evaluation of this system, to support a PSU faculty sabbatical at ERRC, and to fund a Food Science graduate student. Acquisition of the BL-2 facility will give ARS a unique research tool for improving the microbiological safety of fresh produce. A research associate, supported by a Fund for Rural America grant, was hired to investigate sources of contamination of apples with human pathogens. Mature and immature apples from various locations were examined for evidence of internal or external bacterial contamination. A decay-causing fungi, *Glomerella cingulata,* permitted growth of *E. coli* O157:H7 in inoculated apples, probably due to the decrease in acidity that resulted from fungal growth, clearly demonstrating the potential risk of using decayed apples for production of unpasteurized cider. Research on the microbiology of fresh and fresh-cut cantaloupe has provided information about attachment and survival of bacteria including surrogates of human pathogens on external melon surfaces, efficacy of washing treatments in decontaminating cantaloupe melons, and

effective treatments for extending the shelf-life of fresh-cut cantaloupe. These studies are providing a foundation for the development of treatments to assure the microbiological safety of this commodity.

Summary 2000 Accomplishments

Conditions for improving the efficacy of hydrogen peroxide treatments by mechanical detachment of adhering bacteria and by improving contact between the attached bacteria and wash solution were identified, resulting in a 10-fold improvement in population reductions over those obtained previously. Renovations to a Biosafety Level (BSL)-2 pilot plant area, designed for this purpose, were completed, and produce handling and washing equipment designed and fabricated at PSU was installed. This facility will be a valuable national resource in developing effective decontamination technology for fresh produce. Fourteen U.S. orchards were surveyed to determine the incidence and prevalence of *E. coli* O157:H7, and other microflora, including generic *E. coli* bacteria. Though *E. coli* O157:H7 was not isolated from any sample during the study, risk factors associated with the presence of generic *E. coli* on fruit and in environmental samples were identified. These factors should be taken into consideration when compiling a HACCP plan for the production of unpasteurized apple cider. In a one-year study carried out with a cooperating cider producer, incoming fruit and water in which the apples are dumped were examined monthly for presence of *E. coli*. *E. coli* levels varied greatly and peaked during the summer months. Information derived from this study enabled the investigators to track a shipment of heavily contaminated fruit to its source and then to identify specific risk factors for contamination within the originating orchards. Cantaloupe melons were artificially contaminated with non-pathogenic *E. coli* and *Salmonella stanley*, a human pathogen, and survival of these bacteria on melon rind during washing and their transfer to the melon flesh during fresh-cut processing were measured. As the time interval between contamination and washing increased, chlorine and hydrogen peroxide solutions became progressively less effective in reducing contaminant populations, and surviving bacteria were transferred to the flesh where growth occurred. These results demonstrate the importance of developing more effective means of decontaminating whole melons and of suppressing bacterial growth on cut surfaces.

Projected Research Accomplishments During Next 3 Years:

During 2001, the feasibility of surface pasteurization of apples with hot water will be determined in-house by a research microbiologist, a PSU professor on sabbatical at ERRC, and a newly hired mechanical engineer. Produce handling equipment provided by PSU including a dump tank, brush washers, and a dip tank will be validated. A prototype containment structure, provided with a steam decontamination system, will be developed and tested by PSU. Cross contamination of apples with *E. coli* during dump tank operations and performance of produce washing equipment with conventional anti-microbial agents will be investigated using representative commodities. A surrogate organism for *E. coli* O157:H7, required for use in pilot-plant scale washing trials prior to completion of system containment will be selected, based on growth profiles, heat resistance, attachment to and removal from apples and cell membrane fatty acid composition. In a field study

conducted jointly with the University of Illinois, immature and mature apples grown in an orchard adjacent to an active pasture will be evaluated for presence and internalization of *E. coli* O157:H7 and other *E. coli* strains to document modes of contamination of the fruit. Research on cantaloupe will focus on treatments to reduce bacterial pathogen populations on cantaloupe external surfaces. During 2002, research will provide information on performance of produce washing equipment with novel anti-microbial agents and efficacy of sequential treatments with anti-microbial agents.

Novel means of applying anti-microbial agents will be investigated and, if efficacious, scaled up for evaluation in the BSL-2 pilot plant. Promising treatments will be evaluated with additional commodities and modified as required. A full-scale containment and equipment decontamination system will be fabricated and validated. Technology transfer activities will be initiated through contacts with equipment manufacturers and the produce and fresh-cut industries. During 2003, the efficacy of new anti-microbial treatments will be confirmed in a fully contained processing line using human pathogens instead of surrogates. Novel treatments and equipment will be field-tested and treatment costs established. Technology transfer will be actively pursued.

Technology Transfer:
Information on efficacy of conventional and experimental washing formulations in decontaminating apples and on potential hazards in cider production was conveyed to growers, packers, processors, the FDA, and the scientific community through communications with the U.S. Apple Association and participation in meetings sponsored by the Ohio Fruit and Vegetable Growers Congress, the Institute of Food Technologists, International Fresh-cut Produce Association, Regional Project S-294, and the Governments of Canada and Mexico.

PUBLICATIONS:

Annous, B.A., Sapers, G.M., Mattrazzo, A.M., and Riordan, D.C.R. Efficacy of washing with a commercial flat-bed brush washer, using conventional and experimental washing agents, in reducing populations of *Escherichia coli* on artificially inoculated apples. J. Food Protection. In press.

Riordan, D.C.R., Sapers, G.M., and Annous, B.A. 2000. The survival of *E. coli* O157:H7 in the presence of *Penicillium expansum* and *Glomerella cingulata* in wounds on apple surfaces. J. Food Protection 63(12):1637-1642.

Sapers, G.M., Miller, R.L., Jantschke, M., and Mattrazzo, A.M. Factors limiting the efficacy of hydrogen peroxide washes for decontamination of apples containing *Escherichia coli*. J. Food Science. 2000. v. 65. (3). p. 529-532.

Sapers, G.M., Miller, R.L., Pilizota, V., and Kamp, F. 2001. Shelf-life extension of fresh mushrooms (*Agaricus bisporus*) by application of hydrogen peroxide and browning inhibitors. J. Food Science. In press.

Sapers, G.M., Miller, R.L., Pilizota, V., and Mattrazzo, A.M. 2001. Anti-microbial treatments for minimally processed cantaloupe melon. J: Food Science. In press.

PROCEEDINGS/ABSTRACTS:

Riordan, D.C., Sapers, G.M., and Annous, B.A. A survey of U.S. orchards to identify potential sources of *Escherichia coli* O157:H7. Presented at 2000 An. Mtg. of Internat. Assoc. of Food Protection, Aug. 6-9.

Sapers, G.M. Safety aspects of fresh-cut fruits. Abstract 71-2. Presented at Symposium on Fresh-cut Fruit: Factors Affecting Industry Growth, 2000 An. Mtg. of Institute of Food Technologists, June 10-14.

Sapers, G.M., Annous, B.A., Miller, R.L., and Mattrazzo, A.M. 2001. Cider safety research advances and new technologies. Proceedings, 2000 Annual Meeting of Michigan State Horticultural Society.

Sapers, G.M., Annous, B.A., Riordan, D.C., Miller, R.L., and Mattrazzo, A.M. 2001. Contamination and washing of cider apples. Proceedings, 2000 Annual Meeting of Michigan State Horticultural Society.

Ukuku, D.O., Pilizota, V., Sapers, G.M., Cooke, P.H., and Soroka, D.S. Changes in surface properties of cantaloupe and population of *Escherichia coli* ATCC 25922 after exposure to chlorine or hydrogen peroxide. Abstract 65A-6. Presented at 2000 An. Mtg. of Institute of Food Technologists, June 10-14.

Ukuku, D.O. and Sapers, G.M. Survival and growth of *Salmonella stanley* on cantaloupe surface: effect of washing treatments and possibility of cell transfer to fresh-cut tissues during cutting practices. Presented at 2000 An. Mtg. of Internat. Assoc. of Food Protection, Aug. 6-9.

CRIS Title: New Chemical, Physical, and Biological Technologies for Decontaminating Sprouting Seed and Produce with Easily Damageable Surfaces
CRIS: 1935-41420-005
Scientists: Fett WF, Liao C-H., Ukuku D, Sapers GM, Hicks KB
Location: Plant Science and Technology Research Unit, ERRC, Wyndmoor, PA
Contact: 215-233-6418 (P), 215-233-6406 (F); wfett@arserrc.gov

Summary Project Aims:

Recent outbreaks of foodborne illness due to contaminated produce including sprouts and fresh-cut cantaloupe have shaken consumer confidence in the safety of produce at a time when consumers are being urged to eat more fruits and vegetables. The viability of the US sprout industry is threatened and no nationally distributed fresh-cut melon is available due to safety concerns and quality issues. Since 1995 there have been fourteen outbreaks in the US due to consumption of contaminated alfalfa, clover and mung bean sprouts resulting in over 1300 culture confirmed cases and two deaths. Most outbreaks have been due to various Salmonella serovars, but two outbreaks were caused by Escherichia coli O157:H7. The sprout-related outbreaks have resulted in 1) a reduced market for sprouts, 2) the closure of several sprout growing facilities due to outbreak related litigation and 3) the need for expensive testing of spent irrigation water for pathogens. Multiple outbreaks due to contaminated cantaloupe have also been reported, the most recent in April/May of this year (2000) with at least 39 individuals sickened with salmonellosis in five states. Current methods for controlling human pathogens on sprouting seed as well as fresh or minimally processed produce are only marginally effective. New and innovative means of sanitizing seed and produce need to be developed. In this project, research is being conducted into new and more effective chemical, physical and biological methodologies for pathogen elimination. In addition, the ecology of human pathogens on fresh and minimally produce is being studied.

Summary Accomplishments During Entire Project:

This is a new CRIS project initiated in February 2000 with 50% of funding from CRIS project 1935-41420-003. Accomplishments previous to February 2000 are listed in the report of that CRIS project. Briefly, treatment of sprouting seed with 20,000 ppm of free chlorine provided by calcium hypochlorite was shown to result in an approximate 4-log reduction of human bacterial pathogens. This information was used in support of successful petitions to the US and California EPA for temporary approval of the use of this treatment for sanitizing sprouting seed. The method was adopted in FDA guidance to the sprout industry and recently received a permanent registration from the US EPA. No further outbreaks of food borne illness have occurred due to consumption of contaminated sprouts when grown from seed treated in this manner. Further research carried out in collaboration with the Food Safety Research Unit demonstrated that the target 5-log reduction of Salmonella while maintaining seed viability can be achieved by the combination of irradiation with gamma rays (1 kGy) followed by treatment with chlorine (20,000 ppm). This combination treatment of seed can be used to ensure the safety of sprouts consumed in the raw state.

Bacterial human pathogens were found to survive on the surfaces of laboratory-inoculated cantaloupe for prolonged periods during storage under refrigeration. Under conditions of temperature abuse these pathogens actually increased in numbers. Common sanitizers (chlorine and hydrogen peroxide) were found to be less effective for decontaminating the surfaces of cantaloupe as the time between inoculation with bacterial human pathogens and treatment with sanitizer increased indicating the need to develop new, more effective sanitizer treatments, especially since results indicated that pathogens could be transferred from the external surface to the internal flesh during preparation of fresh-cut pieces. Bacterial human pathogens bound to cut surfaces of green pepper were demonstrated to be more difficult to kill with sanitizers than those present on intact surfaces. The interaction of bacterial human pathogens with the natural microflora on produce surfaces has been demonstrated to be highly complex with some naturally occurring bacteria able to inhibit the growth and survival of the pathogens.

Summary 2000 Accomplishments:
Treatment of laboratory inoculated seed with 20,000 ppm of free chlorine was previously shown to result in a 3 to 4 log reduction of human pathogens, but no data was available for naturally contaminated seed. Tests of the efficacy of this treatment on alfalfa seed naturally contaminated with Salmonella mbandaka were accomplished. This method was demonstrated to be effective in sanitizing the naturally contaminated seed. As a result of these studies, growers, consumers and regulators will have greater confidence in the safety of sprouts grown from seed treated in this manner. As previous experiments in our laboratory demonstrated that addition of antimicrobials to irrigation water is ineffective in controlling growth of bacteria on growing sprouts we determined if this might be due to the formation of microbial biofilms. A variety of types of sprouts were examined by scanning electron microscopy. Biofilms consisting of the natural microflora were found to be abundant on all types of sprouts. This information will aid in the development of novel antimicrobial treatments for controlling pathogen growth during the sprouting process. The fate of human pathogens in manure spread on fields used to grow produce is not known. In cooperation with ARS scientists at Orono, Maine, survival of Listeria monocytogenes and Escherichia coli found in liquid dairy manure after application to experimental potato fields was studied. Both bacteria were detected in soil for up to six weeks after addition of manure, but were undetectable at the end of the growing season and in harvested tubers. This work provides producers and regulatory agencies with information useful for developing good agricultural practices.

Projected Research Accomplishments During the Next 3 Years:
Expected accomplishments in 2001 include; complete evaluation of synthetic chemicals for decontamination of sprouting seed; complete libraries of endogenous microflora on sprouts and other produce; and complete bioassays of the natural microflora of sprouts for efficacy in the competitive exclusion of bacterial human pathogens. During 2002: complete studies on the use of natural plant antimicrobial compounds and various physical methods for decontamination of sprouting seed; complete studies on the use of synthetic antimicrobials and various physical methods for decontamination of fresh-cut cantaloupe; complete studies on the interaction between resident microflora including plant pathogens with bacterial human pathogens on produce; and complete the

evaluation of competitive exclusion for inhibiting the growth of human pathogens on sprouts. In 2003: optimize effective antimicrobial treatments for sprouting seed and fresh-cut cantaloupe; complete studies on the effect of the natural microflora on produce on the detection of bacterial human pathogens; and transfer new technologies to end users by presentations to growers and trade associations and by publications

Technology Transfer:
Information on decontamination technologies for sprouting seed and the surface of cantaloupe was conveyed to US sprout growers and scientists by presentations at the annual meetings of the International Sprout Growers Association (ISGA), the International Association for Food Protection, the Institute of Food Technologists, by visiting sprout and produce growing and processing operations, by attendance at the bi-annual board meetings of the ISGA, and by ongoing communications with the US FDA and the California Department of Health Services.

PUBLICATIONS:

Liao C-H, Sapers GM. Attachment and growth of Salmonella chester on apple fruits and in vivo response of attached bacteria to sanitizer treatments. Journal of Food Protection July 2000. v. 63 (7). p. 876-883.

Fett WF. Naturally occurring biofilms on alfalfa and other types of sprouts. Journal of Food Protection May 2000. v. 63 (5). p. 635-632.

PROCEEDINGS/ABSTRACTS:

Fett, W. F. Sanitizing laboratory inoculated seed and naturally contaminated seed with chemicals. Sprout Summit, November 15-16, 1999, National Center for Food Safety and Technology, Summit-Argo, IL

Fett, W. F. US and international research on means of ensuring the microbial safety of sprouts. International Sprout Growers Association Annual Convention, July 6-8, 2000, Vancouver Canada.

10.8

CRIS Title: Decontamination of Alfalfa Seeds and Sprouts with Ozone
CRIS: 1935-41420-005
Scientists: Fett WF
Location: Plant Science & Technology Research Unit, ERRC, Wyndmoor, PA
Contact: 215-233-6418 (P); 215-233-6406 (F); wfett@arserrc.gov

Summary Project Aims:
Recent outbreaks of food borne illness due to consumption of raw, contaminated sprouts have shaken consumer confidence in the safety of sprouts. Since 1995 there have been fourteen food borne outbreaks in the US due to contaminated alfalfa, clover and mung bean sprouts. These outbreaks have resulted in over 1300 culture confirmed cases and two deaths. The majority of the outbreaks were caused by various Salmonella serovars, but two were due to contamination with Escherichia coli O157:H7. In addition, sprouts from one grower were recalled due to contamination by Listeria monocytogenes, although no illnesses were attributed to consumption of these sprouts. Both Salmonella and E.coli O157:H7 can cause serious illness and death especially in the very young, old and immunocompromized. The outbreaks have resulted in 1)a reduced market for sprouts, 2)the closure of several sprout growing facilities due to outbreak related litigation and 3)the need for expensive testing of spent irrigation water for pathogens. The primary source of the contamination has been identified as the sprouting seed. New, more effective methods for decontaminating seeds and sprouts are urgently needed to ensure their safety. A collaborative research program on the use of ozone for this purpose began on July 1, 2000. This effort involves Pennsylvania State University (Dr. Ali Demirci), the University of Georgia (Dr. Larry Beuchat) and ARS with funding provided by National Alliance for Food Safety.

Summary Accomplishments During Entire Project:
This is a new project directly related to new (initiated in February 2000) CRIS project no. 1935-41420-005. Under project -005, a seed soak with 20,000 ppm of free chlorine for 10 minutes was found effectively sanitize alfalfa seed naturally contaminated with a bacterial human pathogen. This seed treatment method is currently recommended to sprout growers by the US FDA. However, equally or more effective alternatives to the use of extremely high levels of chlorine need to be identified as the use of such high levels of chlorine is not desirable due to worker safety and environmental concerns.

Projected Research Accomplishments During the Next 3 Years:
This project is funded for two years. Expected accomplishments for 2001 are: complete studies on elimination of E. coli O157:H7 and L. monocytogenes from seeds by use of ozone; and complete preliminary studies on the effect of ozone on bacteria contained in biofilms on sprouts. In 2002: complete studies on elimination of E. coli O157:H7 and L. monocytogenes from sprouts by use of ozone; complete studies on the effect of ozone on bacterial biofilms on sprouts; and transfer novel decontamination methods to the sprout industry.

CRIS Title: Microbial safety of fresh fruits and vegetables
CRIS: 1275-42000-002
Scientists: Bhagwat AA, Conway WS, McEvoy J, Wachtel M, Vacant (Food Technologist)
Location: Produce Quality and Safety Laboratory, PSI, BARC, Beltsville, Maryland
Contact: 301-504-5106 (P): 301-504-5107 (F); bhagwata@ba.ars.usda.gov

Summary Project Aims:
Contamination of fresh fruits and vegetables with microorganisms pathogenic to humans has increased in recent years. We need to understand how such contamination occurs, how the human pathogens survive and grow on fresh produce and how we can better devise postharvest systems for eliminating or controlling the growth of human pathogenic microorganisms while maintaining the quality and shelf-life of fresh and fresh-cut produce. The objectives will be achieved through devising better detection methods for pathogens, microbial genome analysis, and developing biocontrol agents

Summary Accomplishments during Entire Project:
This is a new project; research has just been started.

Summary 2000 Accomplishments:
Foodborne human pathogens are a potential problem for the rapidly growing fresh-cut industry. We have conducted cooperative research with a bacteriophage-producing company to determine if bacteriophage could provide biological control of *Salmonella enteritidis* on fresh-cut fruits and vegetables. We found that the bacteriophage mixture reduced the populations of *S. enteritidis* on cantaloupe and cucumber. The fresh-cut industry will use this technology to reduce the possibility of foodborne pathogen outbreaks and reduce their dependency on chemical control.

Microbial pathogen detection methods based on Polymerase Chain Reaction (PCR) can not differentiate dead vs. live cells, thus samples may be reported as positive for the presence of pathogens and yet no live bacteria can be isolated. We determined that RNA based PCR methods have the potential to eliminate false positive results. Protocols enabling simultaneous detection of one to four RNA targets from *Salmonella typhimurium* were developed. This method could be a part of a pathogen monitoring procedure to reduce the possibility of reporting false positive detection.

Pathogenic strains isolated from outbreaks are often different from laboratory "type" strains. *Salmonella* and *E. coli* O157:H7 strains implicated in recent outbreaks associated with fresh-cut produce were collected. The data from genetic and biochemical comparisons of the outbreak strains and standard laboratory strains will define unique growth and survival characteristics. The analysis will enable us to design appropriate control strategies and enhance the safety of our food supply.

Projected Research Accomplishments during Next 3 Years:
We are in the final stages of hiring a Food Technologist to interface between the basic microbiology research we are conducting and the commercial situations encountered by the fresh produce industry, particularly with regard to fresh-cut preparation. The Food Technologist will be looking at packaging and other postharvest handling systems in relation to minimizing attachment, survival and growth of foodborne pathogens on fruits and vegetables.

Briefly, we plan to study the ability of human pathogenic bacteria, including *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* spp., to contaminate and grow on fresh fruits and vegetables, including sprouts. To study the attachment and internalization of enterohemorrhagic *E. coli* to fesh cut lettuce and apples a bank of transposon mutants has been created. This mutant collection will be screened for defects in attachment to and survival within fresh cut lettuce and apples. Genes encoding factors involved in bacterial attachment and survival will then be identified and characterized with the goal of blocking these processes. Subsequently, we will devise technologies for rapid detection of pathogens and how to limit growth of these microorganisms without affecting the beneficial microorganisms present. Additionally plans are to develop cost effective postharvest treatments, which will ensure microbiological safety while maintaining product quality, and to provide technical support for regulatory approval of such postharvest system treatments.

Technology Transfer:
Initiated a CRADA with Intralytix, Inc., entitled "The effect of bacteriophage on foodborne pathogens of fruits and vegetables." Intralytix is providing $15,000 for the cooperative research which is 1 year in duration. A second CRADA was initiated with Zylux Corporation, entitled "Optimization of real time detection of residual microorganisms on fresh-cut fruits and vegetables." Zylux Corporation is providing $10,000 for the research which is of 1 year in duration.

PUBLICATIONS:
Conway, W.S., Leverentz, B., Saftner, R., Janisiewicz, W.J., Sams, C.E., and Leblanc, E. Survival and growth of *Listeria monocytogenes* on fresh-cut apple slices and its interaction with *Glomerella cingulata* and *Penicillium expansum*. 2000. Plant Disease 84:177-181

PROCEEDINGS/ABSTRACTS:

Bhagwat, A.A., Mithofer, A., Hotchkiss, A., Gross, K.C., Samadani, R., Ebel, J., Keister, D.L. Microbial dilemma in symbiosis: to suppress or avoid host defense? Cold Spring Harbor Symposium "Microbial pathogenesis and host response". 1999. p. 188. New York.

Purushottam Gawande, Ji Heun Hong, Kenneth C. Gross, and Arvind A Bhagwat. Survival of human pathogens on fresh-cut fruits and vegetables: Genome wide analysis Poster presentation at the Gorden Research Conference on Microbial Stress Responses, Rhode Island (July, 2000).

CRIS Title: Adhesion and Control of Human Pathogens to and on Surfaces: (Part B: Produce)
CRIS: 5325-42000-022
Scientists: Mandrell RE, Charkowski AO, Cooley MB, Friedman M, Gorski L, Kint S, Miller WG
Location: Food Safety and Health Unit, WRRC, Albany, CA.
Contact: 510-559-5610 (P); 510-559-6162 (F); mandrell@pw.usda.gov

Summary Project Aims:
Bacterial contamination of fruit and vegetables is an increasing problem in the United States, and recognition of this problem by the American public is gaining. However, very little is known about how pathogenic bacteria exist in food environments. Both basic and applied research is needed to increase our understanding of how these pathogens attach to and survive in environments related to food, including the soil, water, air, plant roots and leaves, and processing environments. Our work has concentrated on the development of: (a) new detection methods for the identification of pathogens on foods; (b) attachment models to better understand the attachment process of several pathogens to food surfaces, including sprouts, lettuce, cilantro and fresh-cut products;(c) screening for natural antimicrobials that may be added to foods; and (d) the development of new strategies to prevent or minimize bacterial contamination during growth and harvesting of produce.

Summary Accomplishments During Entire Project:
We have developed reagents for the detection of Campylobacter in foods; stable fluorescent Salmonella, Campylobacter, E. coli O157:H7, Listeria monocytogenes and plant bacterial strains for studies of the bacterial ecology and biology in complex environments and biofilms; and attachment assays to study pathogen interactions on surfaces. These assays will provide a quantitative means to measure pathogen reduction on produce, and will enable the characterization of specific gene products that can be targeted for anti-adherence strategies. In addition, our work on biofilms should lead to a more thorough understanding of the natural attachment process of pathogens on a variety of produce items. We anticipate that these studies will lead to the identification of non-toxic methods to decrease pathogens in real food environments.

Summary 2000 Accomplishments:
We demonstrated that some of the chemicals used for sanitizing seeds for sprouting are ineffective against the bacteria and/or they damaged the seeds leading to lack of sprouting. We demonstrated that wrinkled and damaged seeds carry a higher level of bacteria than undamaged seeds and that these seeds are more difficult to sanitize. This work has important implications for the sprout industry and points to potential interventions to minimize pathogens in sprouts. New methods for testing sprout irrigation water are needed to decrease the time for results and the cost. We tested commercially available immunomagnetic beads and determined that they can be

used to recover several different S. enterica strains, all of which were originally isolated from alfalfa seeds, from dilute bacterial suspensions. Initial studies of the ecology of Salmonella on sprouts, E. coli on lettuce, Salmonella on cilantro, Listeria on a variety of cut produce items.

Projected Research Accomplishments During Next 3 Years:
Identify multiple genes related to E. coli attachment to lettuce, Salmonella and E. coli attachment to sprouts, and Salmonella attachment to cilantro leaves. Characterize Campylobacter signaling pathways and relationship to biofilm formation and attachment. Develop assays for assessing chemical and structural basis for attachment of pathogens to plants. Identify plant antimicrobials useful for minimizing pathogens in produce samples. Develop sorting method for testing sprout seed lots. Develop fast method for identifying pathogen in sprout irrigation water. Determine whether pathogen contamination pre-harvest increases resistance of pathogen to interventions; identify some plant genes important in pathogen attachment. Develop improved methods for isolating multiple pathogens from naturally contaminated produce using new reagents and knowledge gained from basic studies. Determine factors crucial to human pathogen fitness and survival on produce. Conduct preliminary studies of pathogen interactions with soil organisms and pathogen fitness. Develop a comprehensive model for how a human pathogen in manure, water, and/or soil colonizes a plant leaf. Establish methods for identification of enteroviruses present in manure, water and on produce. Develop prototype wash solution compatible for multiple produce items.

Technology Transfer:
Technology transfer is an active part of the current CRIS project. Information and technology will be transferred to industry, government regulators, and to the scientific community via publications, collaborations, CRADA's, and Trust Agreements with industry. We continue to seek partners for licensing anti-pathogen antibodies produced in our labs.

PUBLICATIONS:

Kimura, R., Mandrell, R.E., Galland, J.C., Hyatt, D., Riley, L.W. Restriction-site-specific PCR as a rapid test to detect enterohemorrhagic Escherichia coli O157:H7 strains in environmental samples. Applied Environmental Microbiology 2000; v. 66(6). p.2513-9.

Alfano, J. R., Charkowski A. O., Deng W. L., Badel J. L.,Petnicki-Ocwieja, T.,van Dijk, K.,Collmer, A. The Pseudomonas syringae Hrp pathogenicity island has a tripartite mosaic structure composed of a cluster of type III secretion genes bounded by exchangeable effector and conserved effector loci that contribute to parasitic fitness and pathogenicity in plants. Proceedings of the National Academy of Science USA. 2000. v. 97(9). p.4856-61.

Collmer, A., Badel J.L., Charkowski, A. O., Deng, W. L., Fouts, D. E., Ramos, A. R., Rehm, A. H., Anderson, D. M., Schneewind, O., van Dijk, K. Pseudomonas syringae Hrp type III secretion system and effector proteins. Proc Nat Acad Sci USA 2000.v. 97(16).p.8770-8777.

Licking, E., Gorski, L., Kaiser, D. A common step for changing cell shape in fruiting body and starvation-independent sporulation of Myxococcus xanthus. J. Bacteriology; v. 182. p.3553-3558.

Gorski, L., Gornewold, T., Kaiser, D. A sigma-54 activator protein necessary for spore differentiation within the fruiting body of Myxococcus xanthus. J. Bacteriology. 2000. v.182. p.2438-2444.

Cooley, M. B., Pathirana, S., Wu, H. J., Kachroo, P., Klessig, D. F. Members of the Arabidopsis HRT/RPP8 family of resistance genes confer resistance to both viral and oomycete pathogens. Plant Cell. 2000. v.12. p.663-676.

Miller, W.G., Bates, A.H., T, H.S., Brandl, M.T., Wachtel, M.R., and Mandrell, R.E. 2000. Detection of *Campylobacter jejuni* cells transformed with new *gfp*, *yfp* and *cfp* marker plasmids on surfaces and in Caco-2 cells Applied and Environmental Microbioogy. V.66 (12). In press.

PROCEEDINGS\ABSTRACTS:

Brandl, M.T., and Mandrell, R.E. Ecological studies of *Salmonella* serotype Thompson on cilantro plants support its role in recent epidemics. Ann. Meeting of American Society for Microbiology. Los Angeles, CA. 2000.

Brandl, M.T., and Mandrell, R.E. 2000. Use of confocal microscopy and the green fluorescent protein in ecological studies on *Salmonella* on plant surfaces. Scanning 2000. 22p. 83.

Harden, L.A., Lieberman, A., Mandrell, R., and Haddon, W.F. A spreadsheet approach to bacterial identification based on MALDI-TOF spectra of whole cells. American Society Mass Spectrometry, Long Beach, CA. 2000.

Mandrell, R.E., Harden, L., Horn, S.T., Haddon, W.F., and Miller, W.G. Analysis of *E. coli* environmental and diarrheal isolates by MALDI-TOF mass spectrometry: Identification of potential biomarkers ions and a mutation in a gene encoding a biomarker ion. Ann. Meeting of American Society for Microbiology. Los Angeles, CA. 2000.

Wood, D.F., Mandrell, R., Bates, A.H., and Yu, P.C. 2000. Immunolocalization of surface antigens on *Campylobacter jejuni* using FESEM and a backscatter electron detector Scanning 2000. 22p. 79-80.

10.14

CRIS Title: Treatment of Animal Manure to Prevent Pathogen Transmission.
CRIS: 5325-42000-023
Scientists: Ravva SV, Duffy B, Mandrell RE.
Location: Food Safety and Health Research Unit, WRRC, Albany, CA
Contact: 510-559-6176 (P); 510-559-6162 (F); subba@pw.usda.gov

Summary Project Aims:
Basic and applied research to help understand the biology, ecology and control of food-borne pathogens in manure, water and air. Project areas include: develop methods for tracking pathogens in manure, compost, soils, irrigation water and aerosols, identifying mechanisms crucial to pathogen survival in manure, and development of. new strategies for minimizing pathogens in manure and environments influenced by manure.

Summary Accomplishments During Entire Project:
Our group has developed a number of research directions related to understanding the biology of pathogens in food and environments crucial to food production. These include production of reagents for detection of pathogens, pathogen reporter strains for studies of gene regulation, assays for measuring and tracking pathogens in food, identification of non-toxic anti-microbials, methods for fast and sensitive identification of pathogens, and the role of biofilms in pathogen survival. It is anticipated that innovative methods for detecting pathogens in manure and other environments will be developed, and new methods for minimizing pathogens in the environment will be produced

Summary 2000 Accomplishments:
We are designing experiments to address the detection and survival of pathogens in manure and environments influenced by manure. MOU's are being established for monitoring pathogen transport and survival during swine waste processing (Superior Resources, CA) and nutrient management by aeration of dairy waste ponds (Natural Aeration Inc., WA).

Projected Research Accomplishments During Next 3 Years:
Develop methods for enriching, selecting and detecting bacterial pathogens in complex environments related to manure, water and compost. Develop methods for recovering and sampling pathogens in bioaerosols. Develop immunofluorescence microscopy methods for visualizing pathogens in complex samples. Produce reagents and surrogate pathogen strains for tracking pathogens in manure and environments influenced by manure. Determine the survival characteristics of multiple human pathogens in these environments. Screen for novel anti-pathogen compounds. Initiate field studies of surrogate pathogens in manure and compost. Determine the life cycle of human pathogens transported from manure to soils and fruit and vegetable crops. Develop a prototype strategy for minimizing pathogens in manure and crops treated with manure and compost.

CRIS Title: Prevent Zoonotic Pathogen Transmission and Improve the Value of Dairy Manure to the Environment
CRIS: 1265-31420-001
Scientists: Millner PD, Karnes JS, (vacant)
Location: Animal Waste Pathogen Laboratory, ANRI, BARC, Beltsville, MD
Contact: 301-504-8163 (P); 301-504-8370 (F); pmillner@asrr.arsusda.gov

Summary Project Aims:
This project is aimed at improving the safety and value of livestock manure as an environmental amenity through reducing and/or eliminating the risk of human pathogen transmission to the environment via livestock manure and manure compost. Research studies will be designed to develop technologies for minimizing and/or eliminating bacterial pathogens of zoonotic importance which may enter the environment as a result of dairy production practices, and identifying and modifying those components of the dairy production enterprise most likely to negatively impact the environment through pathogen and excessive

Summary Accomplishments During Entire Project:
Key accomplishments include focusing the project efforts on microbial ecology including the digestive tract habitat for pathogen proliferation, and waste management & processing interventions in pathogen destruction. Reducing the environmental burden associated closely with dairy cows and assessing and developing control strategies for eliminating entry mechanisms to the milk supply form the key initiatives undertaken.

Summary 2000 Accomplishments:
The utility of sodium carbonate for pathogen reduction in animal waste was assessed. Preliminary experiments indicated that there was increased ammonium-nitrogen volatilization when manure was treated with sodium carbonate to reduce microbial pathogens. More work is required to determine optimal conditions that result in maximal microbial reduction and minimal nitrogen loss. Over the last year, microbiological methods for identification and enumeration of pathogens were established in a newly formed microbiology laboratory. Based on our research and our assessment of related research to date, we can conclude that many factors can affect the dairy environmental pathogen load. For example, diet has been shown to have an impact on pathogen shedding and survival, but more research is needed to determine the specific effects of animal nutrition. Other critical factors may include environmental conditions, management techniques such as manure handling, milking techniques, or dairy equipment sanitation.

Projected Research Accomplishments During Next 3 years:
Experiments will be designed and conducted to address factors that reduce the environmental impact of dairy production, particularly as related to zoonotic pathogens and excess nutrients in manure. Research will emphasize the biology of dairy production and consider feeding and animal management strategies that impact both digestive and post-digestive processes.

Initial projects will focus on manipulation of the conditions in the rumen and large intestine in order to elucidate key factors that minimize or reduce pathogen shedding. Research will also assess methods of reducing the pathogen load in stored manure.

Technology Transfer:
Preliminary results have been reported at field days and producer meetings such as the 2000 Sustainable Agriculture Field Day at BARC. Technical presentations were made at professional meetings and workshops. Research into the scale of milk pathogen related illnesses and role of milk processing, and focus for our program were presented at a May 2000 FDA/ARS workshop on milk pathogens.

PUBLICATIONS:

Gagliardi JV, Karns JS Leaching of Escherichia coli O157:H7 in diverse soils under various agricultural management practices. Applied Environmental Microbiology. 2000. 66(3): 877-883.

CRIS Title: Reducing the Pathogen Incidence and Contamination Potential of Spent Poultry Litters
CRIS: 6612-42000-027
Scientists: Line JE, Siragusa G, Hiett K, Stern NJ
Location: Poultry Microbiological Safety Research Unit, Athens, GA
Contact: 706-546-3522 (P); 706-546-3771 (F); eline@ars.usda.gov

Summary Projects Aims:
Together, *Salmonella* and *Campylobacter* are responsible for the majority of food borne bacterial infections in the United States. Poultry is the dominant animal reservoir for these bacteria. Poultry litter or manure is known to be a source of these pathogens. This CRIS project will seek to understand the impact of spent poultry litter as both a reservoir and vehicle for transmitting the human pathogens *Salmonella* spp., *Campylobacter* spp. and *Clostridium perfringens*. This project will investigate the extent of the problem (by creating a pathogen-in-litter database) and develop intervention methods to decrease or eliminate pathogen survival in litter to preclude their dissemination to crops, water supplies or to new flocks.

Summary Accomplishments During Entire Project
None to report.

Summary 2000 Accomplishments
As this is a new CRIS, efforts have been limited to the recruitment of a newly assigned ARS Research Microbiologist (Dr. G.R. Siragusa). In turn Dr. Siragusa has begun setting up laboratory facilities and hiring technical support.

Projected Research Accomplishments During Next 3 Years
To develop a pathogen-in-litter database from commercial poultry operations and simultaneously ascertain method efficacy for pathogen detection from poultry litter. This research will result in peer reviewed scientific publication(s) on the pathogen incidence in litter determined with existing cultural methodologies compared to improved methods. Continue database development from commercial poultry operations including genotypic characterization of isolates. Determine factors affecting pathogen survival in poultry litter in order to initiate assessment/development of antimicrobial interventions for litter. This research will result in peer reviewed scientific publication(s) on the pathogen incidence in litter including genotypic characterization of major pathogen groups and their relationships. To develop and/or test antimicrobial interventions for poultry litter in either lab model systems or pilot scale trials in poultry isolation facilities located in Watkinsville, GA. This research is expected to result in potential technologies for reducing the incidence of pathogens in litter intended for agricultural dissemination.

CRIS Title: Advanced Techniques for the Analysis of Chemical Residues in Foods
CRIS: 1935-42000-039
Scientists: Lehotay SJ, Schneider MJ, Pensabene JW, Fiddler W
Location: Food Safety Research Unit, ERRC, Wyndmoor, PA
Contact: 215-233-6433 (P); 215-233-6642 (F); slehotay@arserrc.gov

Summary Project Aims:
Nearly all nations have set maximum residue limits for chemical residues in food which are enforced by regulatory agencies using methods of detection. This project addresses the problem related to the lack of rapid, automated, cost-effective, waste-minimizing, safe, and high-quality analytical methods to detect multiple chemical residues in foods. New analytical methods are needed to expand the range of veterinary drug and pesticide analytes that can be detected in animal and plant derived food products in a more efficient process. These needs are being met by the use of modern methods and approaches which are being evaluated to screen, quantify, and/or confirm chemical residues. These advanced technologies include hyphenated gas (GC) or liquid (LC) chromatography/ mass spectrometry (MS) (or tandem mass spectrometry - MS^n), supercritical fluid extraction (SFE), aqueous microdialysis, solid phase extraction (SPE), and other techniques. By providing better methods for risk assessment, increasing laboratory productivity, and potentially reducing the amount of chemical residues in food, the research findings and approaches help improve and maintain a safe food supply. The health of consumers is adversely affected by the presence of harmful chemicals in food, and pesticide and veterinary drug residues in food are a serious concern of consumers in the U.S. and worldwide. Minimization of the time, effort, and cost, and improvement in the quality of the results of these analyses, would provide a significant benefit to governments, industry, academic scientists, and consumers. The implementation of improved analytical approaches will (a) increase productivity and/or lower costs of analysis, (b) provide more statistically valid and accurate results for risk assessment and other purposes, (c) overcome trade barriers associated with the analysis of chemical residues, (d) provide more information to understand the effects and mechanisms of antimicrobial resistance and endocrine disruption, (e) allow for better verification of organic food labeling, (f) improve possible industrial HACCP programs, and (g) reduce the potential for food that has been deliberately or accidentally adulterated by toxic chemicals to reach the consumer.

Summary Accomplishments During Entire Project:
One major accomplishment is the investigation of automated microdialysis - LC as a technique for multiresidue determination of fluoroquinolone antibiotics in eggs and chicken tissue. Increased microbial resistance to antibiotics and in particular to fluoroquinolones has made this an area of great concern, and efficient methodologies for the analysis of fluoroquinolone antibiotic residues in meat and eggs are required to monitor their presence in the food supply. The promise of this new approach lies in the use of aqueous microdialysis for on-line sample cleanup in an automated instrument which minimizes the generation of hazardous waste and reduces manual labor. SFE has also been extensively investigated in a variety of applications involving chemical residues in foods over the course of this project. SFE is a safe, rapid, selective, easy, and waste-minimizing approach to extraction which uses pressurized and heated carbon dioxide to dissolve analytes of interest rather than liquid organic solvents. SFE was used in several applications involving many residues in many sample types. For example, SFE methods were developed for triazines and organochlorine pesticides in eggs, multiple pesticides in eggs, grains, fruits, and vegetables, sulfonamides in liver

and other tissues, fluoroquinolones in chicken tissues and eggs, chloramphenicol in eggs, dioxins in an anti-caking agent in feed, melengesterol acetate in fat, and other applications. A major accomplishment involving SFE was the completion of interlaboratory studies through AOAC International and European Union for the determination of diverse pesticide residues in nonfatty foods. Some of the SFE methods developed in this program are successfully being used in monitoring labs in other countries. In the future, due to the results of these studies, the SFE methods could become more widely used in laboratories around the world that routinely monitor chemical residues in food.

Summary 2000 Accomplishments:
Currently, the analytical method used for pesticide residues in eggs is very laborious, time-consuming. In 2000, the most significant accomplishment was the development and evaluation of a novel approach of rapid analysis for approximately 40 pesticides in eggs using direct sample introduction/GC/MS-MS in support of FSIS needs to monitor pesticide residues. A rapid, sensitive, quantitative, confirmatory, simple, inexpensive, safe, and rugged procedure was developed to monitor pesticide levels as low as 1 ng/g. This approach avoids the costly, time-consuming and labor-intensive clean-up and solvent evaporation steps associated with traditional methods. This approach has the potential to make a strong impact in the analysis of many types of pesticides and other semi-volatile chemicals in a variety of matrices, including food. Also, the method commonly used for the determination of thyreostats, which are banned growth promotants, uses a mercury-column clean-up step which generates mercury waste. A novel multiresidue analytical screening method was developed for the isolation and detection of thyreostatic agents in meat tissue without the need for a mercury column cleanup step. The total amount of solvent used for the analysis is less than 30 mL, and recoveries from fortified meat tissue were consistent and greater than 85% for thiouracil, tapazole, 6-methyl-2-thiouracil, and 6-n-propyl-2-thiouracil. GC/MS-MS conditions were also devised for reliable confirmation of the presence of the drugs in the samples. This method provides an alternative to the current method adopted by FSIS and the European Union which generates more and worse hazardous waste due to the mercury column clean-up step. Also, a modification of the current FSIS method to extend the method to analyze more organophosphorus pesticides in fat has been evaluated. The method uses both a micro-electron capture detector and pulsed flame photometric detector after splitting the flow after the GC column. Only a single inection is still needed to extend the method to many OP pesticides previously not detected which are important for FSIS to meet risk assessment and presticide re-registration needs.

Projected Research Accomplishments During Next 3 Years:
The proposed future research will involve the improved determination and confirmation of fluoroquinolone drugs in animal tissues using LC/MS-MS. Also, the same approach will be used to monitor for beta-agonist growth promoters. The LC/MS-MS approaches will be compared with derivatization methods that using GC/MS-MS. The use of MS approaches should allow for a single multiresidue method for multiple compounds and may also provide quantitation and confirmation of the results simultaneously. Current common methods often detect single or only a few analytes and require separate injections for confirmation of the identity of the chemicals. Upon completion of investigations involving fluoroquinolones and beta-agonists, the next classes of veterinary drugs planned for inclusion in the multiclass, multiresidue approaches are phenicols and beta-lactam antibiotics which have been selected based on FSIS priorities. Rapid SPE procedures will be

devised to provide clean-up of matrix components. In the case of pesticides, research will focus on rapid and simple, laboratory-based methods, such as direct sample introduction, for trace-level pesticide residues in a variety of foods. The goal of this approach is to expand the number of pesticides analyzed to more than 100 and reduce analysis time to 15 min. Furthermore, investigations of novel, portable screening techniques are planned for the detection of multiple chemical contaminants at regulatory tolerance levels.

Technology Transfer:
The results from research studies have been transferred to interested parties by means of collaborations, peer-reviewed publications, written reports, internet accounts, presentations at scientific meetings, and communications with scientists and administrators from regulatory agencies, industry, and other organizations. More directly, the SFE and GC/MS method was conducted among 20 labs in 7 countries as part of a collaborative study. A few of these labs had already implemented the approach, and others are also expected to do so. Once the method achieves Official Method status, a number of other labs are likely to begin using the method routinely. Recommendations designed to meet FSIS residue detection needs for meat, poultry, and eggs have been provided to FSIS. FSIS must decide whether to implement the methodology which involves the purchase of instrumentation, training of personnel, and method validation studies. FSIS laboratories have limited resources and personnel and many other types of analyses to conduct, but it is possible that FSIS will implement the more efficient methods in the future to extend the analytical range of their current methods and increase laboratory productivity.

PUBLICATIONS:

Lehotay, S.J. Analysis of pesticide residues in mixed fruit and vegetable extracts by direct sample introduction/gas chromatography/tandem mass spectrometry. Journal of AOAC International. 2000. v.83 (3). p.680-697.

Schenck, F.J., Lehotay, S.J. Does further clean-up reduce the matrix enhancement effect in gas chromatographic analysis of pesticide residues in food? Journal of Chromatography A. 2000. v.868 (1). p.51-61.

Pensabene, J.W., Fiddler, W. Supercritical fluid extraction of nitrosamines from cured meats. Supercritical Fluid Methods and Protocols. 2000. v.13. p.23-29.

Pensabene, J.W., Fiddler, W., Donoghue, D.J. Supercritical fluid extraction of atrazine and other triazine herbicides from fortified and incurred eggs. Journal of Agricultural and Food Chemistry. 2000. v.48 (5) p.1668-1672.

Pensabene, J.W., Fiddler, W., Donoghue, D.J. Isolation of chloramphenicol from whole eggs by supercritical fluid extraction with in-line collection. Journal of AOAC International. 2000. v.82 (6). p.1334-1339.

Maxwell, R.J., Parks, O.W., Shadwell, R.J., Lightfield, A.R., Henry C., Fuerst, B.S. Supercritical fluid extraction of melengestrol acetate from bovine fat tissue. Supercritical Fluid Methods and Protocols. 2000. v.13. p.31-40.

Hampson, J.W., Maxwell, R.J., Li, S.F., Shadwell, R.J. Solubility of three veterinary sulfonamides in supercritical carbon dioxide by a recirculating equilibrium method. Journal of Chemical and Engineering Data, 1999. v.44 (6). p.1222-1225.

PROCEEDINGS/ABSTRACTS:

Schneider, M. J., Donoghue, D. J. Analysis of fluoroquinolone antibiotics in eggs and chicken tissue using automated microdialysis - liquid chromatography. Proceedings of EuroResidue IV Conference on Residues of Veterinary Drugs in Food. 2000. v.2. p.998-1004.

Lehotay, S. J. AOAC International collaborative study on the determination of pesticide residues in nonfatty foods by supercritical fluid extraction and gas chromatography/mass spectrometry. In *Principles and Practices of Method Validation*, A. Fajgelj and Á. Ambrus (Eds.), Royal Society of Chemistry, Cambridge, UK. 2000. p.89-99.

CRIS Title: Alternate Solvent Systems and Techniques for Food Analysis
CRIS: 3620-42520-001
Scientists: King JW
Location: New Crops Research, National Center Agricultural Utilization Research, Peoria, Il
Contact: 309-681-6203 (p); kingjw@mail.ncaur.usda.gov

Summary Project Aims:
Reduction or total elimination of the use of objectionable organic solvents in the laboratory environment is currently desired by several government regulatory agencies. Aside from the environmental benefit, elimination of the use of these solvents and chemicals would substantially reduce the costs and difficulties associated with the disposal of such substances and, further, protect laboratory personnel from exposure to these toxic, flammable chemicals. In this research program, two natural chemicals, carbon dioxide and water, along with small quantities of organic solvents, are used to extract analytes (pesticides, nutrients, and veterinary drugs) that are routinely monitored by governmental agencies such as the Food Safety Inspection Surface (FSIS) and Food & Drug Administration (FDA). The basic extraction methodology utilized is called supercritical fluid extraction (SFE), and it is capable of extracting the above analytes with similar or better efficiency then the traditional solvent extraction methods. Lack of specificity during the SFE has required the integration of cleanup techniques to remove unwanted coextracted material, leaving the target analytes purified for analysis. One variant of analytical SFE has become quite popular for the determination of fat required in food nutritional labeling claims. Our laboratory and its research have made a major contribution in this area over the past decade.

Summary Accomplishments During Entire Project:
The NCAUR supercritical fluid program has been a major contributor in promoting the analytical use of critical fluids for over a decade. Analytical SFE, due in part to our group's efforts, is becoming widely adopted by many analytical laboratories throughout the world for the extraction of trace levels of pesticides and nutrients in foods. Due to efforts in our laboratory, analytical SFE has become a standard technique for the analysis of fat and oil levels in a number of agricultural and food products. These techniques and methods have freed analysts of the need to use copious amounts of environmentally-harmful solvents and reduced the time and cost of performing such assays. A recent major emphasis of the analytical research and development effort of our group has been focused on the integration of the sample cleanup step into the analysis procedure. This emphasis will continue on within the new CRIS by using such assisting techniques such as solid phase microfiber-, solid phase-, or accelerated solvent-extraction. As opposed to solely using SFE with carbon dioxide as an extraction solvent, pressurized water extraction is being utilized in tandem with the above assisting techniques plus matrix solid phase dispersion in sample preparation prior to extraction. This research program has made generic contributions in the design of instrumentation for conducting analytical SFE, which has resulted in the eventual development, production, and sale of instrumentation based on NCAUR research to the American and international marketplace. Hence, we are routinely consulted by many industrial companies and regulatory agencies on this topic.

Summary 2000 Accomplishments:
Method development and basic research has been initiated on this relative new CRIS, particularly in the area of pressurized water extraction of pesticides from foodstuffs. The solubilities of several pesticides (atrazine, cyanazine, and simazine) were measured in hot water. These measurements provided essential information on the extraction capabilities of compressed water for the target pesticides. Both pure water and water modified with a non-toxic additive were examined. Pesticide solubilities increase with temperature and are sufficient to allow the quantitative extraction of the above analytes. Utilizing this basic data, an extraction and cleanup scheme has been devised for the removal of the pesticides from beef kidney. The co-extraction of interfering matrix components is minimal using this method, allowing chromatographic and/or spectroscopic methods to be applied to quantify the amount of pesticide in food matrices. In collaboration with a visiting scientist from Sweden, an enzyme was used in conjunction with pressurized carbon dioxide for the extraction and sample preparation of vitamin A in several food products, including milk powder, liver paste and minced meat. The presence of the enzyme facilitated the rearrangement of the vitamin A moiety, thereby avoiding a chemical-based and time-consuming sample preparation step. This is an improvement in recently published methods developed by participating European Union laboratories in a collaborative study using carbon dioxide extraction for nutritional vitamin assay in food products. Further refinement of a gravimetric-based proximate method for the determination of the fat content of meats was undertaken this last year. Variables such the fate of water in the meat sample during extraction, optimization of the collection technique, and comparison of the carbon dioxide-based extraction method with total fat assays using organic solvents were reported. The technique which utilizes commercial instrumentation appears to be in the final stages of refinement prior to publishing the method.

Projected Research Accomplishments During Next 3 Years:
A specific request from the FSIS Midwestern Laboratory in St. Louis,Missouri, to couple analytical SFE with a microbial inhibitor test for screening meat tissues for the presence of antibiotic residues has received limited attention by the scientist assigned to the project this past year. Future work will focus on the feasibility of coupling the two techniques for the screening of polyether antibiotic residues in meat samples. Method development will continue on the use of pressurized water as an environmentally-benign extraction media. A focus on the banned antibiotic, avoparcin, will consist of making solubility measurements of this analyte in subcritical water and developing the most appropriate coupling of sample cleanup techniques to yield optimal extraction of this analyte from avoparcin- spiked meat samples. Water extractions will also be conducted on specific carbamate pesticides to test extraction efficiency and analyte stability. This will include the coupling of compressed water extraction with enzyme immunoassays (EIAs), both environmentally-benign techniques that can be performed in aqueous media. A new, analyte specific technique using molecularly imprinted polymers in conjunctin with SFE will be investigated.

Technology Transfer:
Analytical SFE technology developed at NCAUR has been adopted by numerous industrial and Federal/State regulatory laboratories. We have also played a significant role in influencing the design of instrumentation use in this field as attested by instrumentation currently offered by three USA-based instrumentation companies. Research continues with FDA's Total Diet and Pesticide Research Center in Lenexa, KS, on the use of binary fluids for the extraction of pesticides from

fat-containing foods as well as the use of pressurized water toward the same end. Through annual reports, method development technology is transferred to FSIS. This year the Lead Scientist addressed the American Feed Industry Association on the application of supercritical fluids for the analysis of toxicants and nutrients. A team scientist also is providing method development assistance to a major American instrument company on fat analysis of meats and food components. The Lead Scientist also has provided extensive consultation to a Pennsylvania-based company on the use and development of newly designed SFE instruments.

PUBLICATIONS:

King, J.W., Zhang, Z. Theoretical optimization of analyte collection in analytical supercritical fluid extraction. Chromatographia. 2000. p. 467-47

PROCEEDINGS/ABSTRACTS:

Curren, M.S.S., King. J.W. Solubility of triazine pesticides in subcritical water. Proceedings of the 3rd Biennial International Conference on Monitoring and Measuring in the Environment. 2000. p. 411-416.

Turner, C.A., King, J.W., Mathiasson, L. Determination of fat soluble vitamins in food matrices using a lipase-catalyzed reaction coupled with SFE. Proceedings of the 5th International Symposium on Supercritical.

King, J.W., Lee, K., Clifford, A.A. Binary critical fluid mixtures for extraction, fractionation, and reaction chemistry. 14th Symposium on Thermophysical Properties. 2000. Abstract. p. 238.

Taylor, S.L., King, J.W. Fatty and resin acid analysis in tall oil products via SFE/SFR using enzymatic catalysis. Pittsburgh Conference. 2000. Abstract No. 2214P.

King, J.W., Eller, F.J., Taylor, S.L., Snyder, J.M. Analytical SFE: utilization in developing extraction and chromatographic processing involving critical fluids. 38th Annual Eastern Analytical Symposium. 1999.

Eller, F.J., King, J.W. Fat in ground beef: accuracy of supercritical CO2 extraction. American Oil Chemists' Society Meeting. 2000. Abstract p. 42.

Turner, C., King, J.W., Mathiasson, L. Analytical ASE of fat-soluble vitamins in food matrices: off-line saponification vs. on-line enzymatic hydrolysis. American Oil Chemists' Society Meeting. 2000. Abstract p. 72.

11.8

CRIS Title: Dioxins and Other Environmental Contaminants in Food.
CRIS: 5442-42000-002
Scientists: Huwe JK, Shappell NW, Shelver WL, Hakk H, Garber EAE, Larsen GL, Smith DJ
Location: Animal Metabolism-Agricultural Chemicals Research Unit, Red River Valley Agricultural Research Center, Fargo, ND
Contact: 701-239-1288 (P); 701-239-1430 (F); huwej@fargo.ars.usda.gov

Summary Project Aims:
Persistent organic pollutants such as polychlorinated dioxins, furans, and biphenyls are ubiquitous in the environment and enter the food chain as animals are exposed through their surroundings and feed. These toxic contaminants are concentrated in animal products containing fats (i.e. meat, dairy, and eggs) and are ultimately consumed by humans. The health risk to humans of these low-level contaminants is not adequately known. Our research efforts are directed at reducing this exposure from the food supply by 1) determining background levels currently found in domestic animal products, 2) identifying and reducing potential sources of animal exposure to these toxic chemicals, 3) exploring methods which may prevent or lower the accumulation of these chemicals in animal fats, and 4) developing rapid, lower cost methods to analyze food products for these chemicals.

Summary Accomplishments During Entire Project:
A geographical survey of domestic beef showed low levels of dioxins and furans in general, but indicated that when beef samples are contaminated with significant levels of dioxins and furans it was due to pentachlorophenol treated wood in feeding facilities. The absorption and distribution of dioxins and furans in beef cattle was determined through a controlled feeding study and related metabolism studies in calves and model animals. The data indicate that an animal exposed to the toxic dioxins retains them until it is harvested for food. Distribution of dioxins is into the fat and to a higher degree into the liver. Strategies to reduce dioxin intake by humans include trimming fat, cooking food and discarding the fats and juices (which can remove 45% of the dioxins from hamburger as determined by our studies), and avoiding eating liver. An immunoaffinity column has been developed for the isolation of dioxins and furans from serum. This column recognizes seven of the most toxic congeners and has potential to decrease cleanup time, solvent consumption, and cost of dioxin analyses.

Summary 2000 Accomplishments:
Remediation protocols for dioxin contaminated livestock do not exist. At our laboratory we investigated the use of clenbuterol, a leanness enhancing agent, in lowering dioxin body burdens after an exposure. A preliminary study was conducted in rats which showed body fat and dioxin levels decreased by 30% compared to controls when clenbuterol was fed after a dioxin exposure. These results are encouraging and suggest similar body burden decreases could be achieved in livestock. Polybrominated diphenyl ethers (PBDEs), a class of flame retardants, have been found to be increasing in the environment and in marine life; however, no data has been reported for livestock species. In our laboratory, we have begun to investigate the occurrence of PBDEs in food products. Purification methods and GC/MS detection methods were developed and applied to the analysis of 13 chickens fat samples. All the samples analyzed contained PBDEs with levels reaching 45 ng/g lipid in an area near a PBDE production facility.

Projected Research Accomplishments During Next 3 Years:
Investigate the use of our immunoaffinity column for milk samples; to determine distribution and absorption patterns of brominated diphenyl ethers in animals; to examine the effect of clenbuterol on dioxin metabolism in rats. Determine the metabolism and disposition of 1,2,3,6,7,8-HxCDD, an important contaminant in pentachlorphenol formulations, in cattle; to extend the investigation of clenbuterol or ractopamine remediation methods to turkeys; to investigate the role of predioxins in dioxin exposure. Test remediation agents in pigs; to report survey data on the background levels of dioxins, PCBs, and PBDEs in certain meats (chicken and beef).

Technology Transfer:
Our studies on the metabolism of decabrominated diphenyl ether and the detection of polybrominated diphenyl ethers in chickens were reported to scientists from government, industry, and academia at an international meeting. These were the first reports on these topics. Preliminary results from remediation experiments with clenbuterol in rats which had been exposed to dioxins and furans was reported to scientists from government, industry, and academia at an international meeting. Further work is needed to assess whether this remediation strategy will be feasible in farm animals. The use of an immunoaffinity column in the analysis of dioxins and furans has been evaluated in collaboration with scientists from other governmental agencies. Although the technique is promising, commercialization is unlikely due to patent licencing costs. A CRADA is ongoing with a private sector company to evaluate a receptor based assay for inexpensive, rapid dioxin screening.

PUBLICATIONS:

Hakk, H., Larsen, G.L., Örn, U., Bergman, Å. Association of decabromodiphenyl ether with urinary and biliary carrier proteins. Organohalogen Compounds. 2000. v. 49. p. 108-111.

Hakk, Heldur, Larsen, Gerald. Levels of chlorinated dioxin and furan emissions from pentachlorophenol-treated wood. Organohalogen Compounds. 2000. v. 45. p. 348-351.

Hakk, Heldur, Larsen, Gerald L. The effect of 2378-TCDD on the plasma levels of leptin, tryptophan, and lipid parameters. Organohalogen Compounds. 2000. v. 49. p. 197-200.

Hakk, Heldur, Larsen, Gerald L. The effect of preinduction with non-toxic dioxins on the metabolism of the toxic 2378-TCDD. Organohalogen Compounds. 2000. v. 49. p. 205-208.

Huwe, Janice K., Feil, Vernon J., Zaylskie, Richard G., Tiernan, Thomas O. An investigation of the *in vivo* formation of octachlorodibenzo-*p*-dioxin. Chemosphere. 2000. v. 40. p. 957-962.

Huwe, Janice K., Lorentzsen, Margaret, Thuresson, Kau, Bergman, Åke. Polybrominated diphenyl ethers in chickens. Organohalogen Compounds. 2000. v. 47. p. 429-432.

Shappell, Nancy W., Billey, Lloyd O., Feil, Vernon. Effect of clenbuterol on fat stores of dioxins and furans in rats. Organohalogen Compounds. 2000. v. 49. p. 116-119.

Shelver, Weilin L., Huwe, Janice K., Stanker, Larry H., Patterson, Jr., Donald G., Turner, Wayman E. A monoclonal antibody based immunoaffinity column for isolation of PCDD/PCDF from serum. Organohalogen Compounds. 2000. v. 45. p. 33-36.

PROCEEDINGS/ABSTRACTS:

Hakk, Heldur, Larsen, Gerald L., Bergman, Å., Klasson-Wehler, Eva, Ulrike, Örn. Excretion, disposition, and metabolism of 2,2'4,4',5-pentabromodiphenyl ether (BDE-99) in male rats. 3rd SETAC World Congress. 2000. Abstract p. 178.

Huwe, Janice K., Shelver, Weilin L., Patterson, Jr., Donald G., Turner, Wayman E. Isolation of polychlorinated dioxins and furans from serum using immunoaffinity chromatography. American Chemical Society. 2000. Abstract #18. p. 21.

Larsen, Gerald L., Hakk, Heldur, Bergman Å, Klasson-Wehler, Eva, Ulrike, Örn. Excretion, disposition, and metabolism of tetrabromobisphenol-A in male rats. 3rd SETAC World Congress. 2000. Abstract p. 178.

CRIS Title: Absorption, Distribution, Metabolism & Elimination of Veterinary Drugs and Mycotoxins in Food Animals
CRIS: 5442-32000-007
Scientists: Smith DJ, Huwe JK, Shappell NW, Hakk H, Garber EAE, Larsen GL, Shelver WL.
Location: Animal Metabolism-Agricultural Chemicals Research Unit, Red River Valley Agricultural Research Center, Fargo, ND
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Summary Project Aims:
Beta adrenergic agonists such as clenbuterol and salbutamol have a history of being used illegally by some livestock producers for their leanness enhancing effects. Human poisonings have not occurred in the United States, but cases of human intoxication after the consumption of beta-agonist contaminated meat products have been documented in several European countries as well as in Asia. We will determine the absorption, distribution, metabolism and excretion (ADME) of beta-agonists in livestock species for which there may be a food safety hazard from illicit use. An additional objective is to develop immunoassays towards those beta-agonists of potential misuse; these immunoassays may then be used to detect illegal or off-label use. Cattle that graze N. coenophialum infected tall fescue are susceptible to a number of maladies that decrease their productive capabilities. The maladies, which are termed "fescue foot" or "fescue toxicosis", are caused by ergopeptine alkaloids produced by the endophytic fungus *N. coenophialum.* The most abundant alkaloid present in affected plants, ergovaline, has been hypothesized to be the causative agent of fescue toxicosis, but the syndrome has not been replicated using purified materials. In order for toxicity to occur, toxins must be absorbed and must reach target tissues. The rate of toxin elimination through metabolism and excretion -or accumulation due to slow elimination- is fundamental to the understanding of the developments of clinical signs of toxicosis. Thus an understanding of the metabolism and disposition of the major ergopeptine alkaloids such as ergovaline is fundamental to an understanding of fescue toxicity.

Summary Accomplishments During Entire Project:
The current CRIS project was initiated in February, 1999. Accomplishments of this project are itemized below. For the previous CRIS project "Disposition of Beta-Agonists in Farm Animals", project number 5442-32000-006, we described the tissue residues of ractopamine HCl and clenbuterol hydrochloride in several species. These studies also included descriptions of the stereochemical compositions of tissue residues and biotransformation products. We also demonstrated for the first time that the leanness enhancing activity of a phenolic phenethanolamine beta-agonists resides with specific stereoisomers. This work was conducted with in vitro and in vivo studies. We also were able to successfully generate polyclonal antibodies towards phenolic phenethanolamine beta agonists and were able to characterize their specificities and sensitivities.

Summary 2000 Accomplishments:
Ractopamine HCl is a newly approved feed additive for use in swine that has the potential to be used in an off-label manner in a number of agricultural species. A highly sensitive and specific monoclonal antibody was developed at the USDA ARS Biosciences Research Laboratory Animal Metabolism-Agricultural Chemicals Research unit. Binding affinities of the antibody towards

ractopamine, ractopamine analogs, ractopamine metabolites, ractopamine stereoisomers, and other phenethanolamine beta-agonists were determined and the antibody's potential for use in a screening assay was validated. The antibody has potential use in many different types of screening assays that could be used to determine whether off-label ractopamine use has occurred and has generated a interest from regulatory agencies, pharmaceutical companies, and ELISA manufactures. Clenbuterol residues present in edible tissues of hogs have caused intoxications of human consumers of hog products. No data were available on the distribution and elimination of clenbuterol in hogs so a clenbuterol residue depletion study was conducted at the USDA-ARS BRL. Clenbuterol and its metabolites were present in most of the edible tissues in hogs slaughtered with 0, 3, and 7 day withdrawal periods and parent clenbuterol represented a majority of the total residue depending upon the withdrawal period. The inactive clenbuterol stereoisomer predominated in all edible tissues that were measured. This data indicates that the toxic stereoisomer of clenbuterol is not preferentially retained in edible tissues, and that animals given clenbuterol retain residues longer than animals administered other beta agonists. Dioxins are long-lived environmental contaminants for which no remediation methods exist after animal exposures. A study was conducted in which the leanness-enhancing agent, clenbuterol, was administered to rats previously dosed with a mixture of dioxins in order to determine whether beta-agonists have the potential to remediate dioxin contamination. Clenbuterol caused rats to have leaner carcasses than control animals and also decreased dioxin congeners in adipose tissue stores. These are the first data indicating that beta-adrenergic agonists could have potential utility in remediation of dioxin-contaminated animals.

A project was undertaken to determine the biological activities of ractopamine and ractopamine stereoisomers in an in vitro muscle cell model. Muscle cells were grown in culture and were dosed with ractopamine and its individual stereoisomers and variables such as cyclic AMP response were measured. Cellular responses were evoked to the greatest extent by the RR stereoisomer of ractopamine and to a lesser extent by the SR stereoisomer. Results of this study show that ractopamine can act directly on muscle cells evoking a physiological response with one stereoisomer evoking the greatest cellular response. A study was conducted to determine the cross reactivity of several clenbuterol immunoassays to the major bovine biotransformation products of clenbuterol and to purified stereoisomers of clenbuterol. Responses of clenbuterol immunoassays were measured when incubated with purified metabolites and to purified clenbuterol stereoisomers. Immunoassays designed to detect clenbuterol cross-reacted to variable degrees with clenbuterol metabolites and surprisingly to clenbuterol stereoisomers. The data indicate that quantitative measurements derived from clenbuterol immunoassays that have not been validated against clenbuterol metabolites could be inaccurate.

Projected Research Accomplishments During Next 3 Years:
We will conduct the first balance-excretion studies with radiolabeled ergopeptines. Depending upon the amount of radiolabeled material available these studies could be conducted in rodents, small ruminants (sheep or goats), or in cattle. Identification of metabolites will ensue. If radiolabel is not available by this time, we will begin generating target molecules by culture methods. Radiolabeled beta-agonists will be synthesized and we will also endeavor to conjugate haptens of new beta-agonists to carrier proteins. Immunization animals with antigen-hapten complexes to generate antibodies will follow. Animals will be immunized with the hopes of generating polyclonal and

monoclonal antibodies. In vitro studies will be initiated investigating the cellular toxicity of ergopeptines; these studies are preliminary experiments for investigating the transport of ergopeptines by intestinal cells.

The identification or ergovaline metabolites will be completed and studies quantifying the extent of ergopeptine absorption will be initiated. Biliary metabolites will be identified. Validation of new beta agonist immunoassays will continue including measuring cross- reactivity to major metabolites. Metabolites will be generated in species (rodents)for which off-label use of the beta-agonist is likely to occur. Transport of ergot alkaloids in cell models will be studied. Ruminal metabolism of the ergopeptine alkaloids will be studied. Major metabolites will be identified and the ruminal bacterial species responsible for biotransformation will be investigated. Validation of new immunoassays will be completed; the utility of the antibodies for use in "real world" will be assessed by conducting a residue depletion study.

Technology Transfer:
A patent entitled "A Monoclonal Antibody, Cell Line and Immunoassay for Ractopamine", authored by W. L. Shelver and D. J. Smith, was filed by the Office of Technology Transfer; July, 2000 (Pending patent number SN 09/615,298). Research results on the elimination of clenbuterol from swine and the elimination of clenbuterol stereoisomers were presented at the American Chemical Society meetings and at the American Society of Animal Science meetings. At his request, reprints and preprints of clenbuterol residue depletion studies were shared with Dr. Thomas Sit, Senior Veterinary Officer, Veterinary Public Health Office, in Hong Kong. A study on the potential for clenbuterol to remediate polychlorinated dioxin contamination in live animals was shared with scientists at the dioxin 2000 meetings. This presentation was attended by an international audience consisting of industrial, academic, and regulatory chemists.

PUBLICATIONS:

Miller, K. B., J. S. Caton, D. M. Schafer, D. J. Smith, J. W. Finley. High dietary manganese lowers heart magnesium in pigs fed a low-magnesium diet. Journal of Nutrition. Aug. 2000. v. 130 (8). p. 2032-2035

Shappell, N. W., Billey, L. O., Feil, V. Effect of clenbuterol on fat stores of dioxins and furans in rats. Organohalogen Compounds. 2000. v. 49. p. 116-119.

Shappell, N.W., Feil, V. J., Smith, D. J., Larsen, G. L., McFarland, D. C. Response of C_2C_{12} mouse and turkey cells to the b-adrenergic agonist ractopamine. Journal of Animal Science. 2000. v. 78. p. 699-708.

Shelver, W.L., Smith, D.J. Development of an immunoassay for the beta-adrenergic agonist ractopamine. Journal of Immunoassay. 2000. v. 21. p. 1-23.

Smith, D. J. Stereochemcial determination of clenbuterol residues in hogs. Journal of Animal Science. 2000. v. 83(suppl. 1). p. 108.

Smith, D. J, Feil, V. J., Paulson, G. D. Identification and metabolism of turkey biliary metabolites of ractopamine HCl and the metabolism and disposition of synthetic [^{14}C]ractopamine glucuronides in turkeys. Xenobiotica. 2000. v. 30 (4). p. 427-440.

PROCEEDINGS/ABSTRACTS:

Smith, D. J. Depletion of total radioactive residues and clenbuterol residues in swine tissues after dietary administration of [^{14}C]clenbuterol HCl. American Chemical Society. 2000. Picogram No. 58: Abstract No. 96.

CRIS Title: Control of Fusarium Mycotoxins and Diseases in Corn and Small Grains
CRIS: 3620-42000-018
Scientists: Plattner RD, Desjardins AE, Muhitch MJ, Proctor RH
Location: Mycotoxin Research Unit, NCAUR, Peoria, IL
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Summary Project Aims:
Some fungi can infect our crops while they are growing in the field. In the process, they make toxins that are harmful to humans and animals that eat contaminated food or feed from these crops. In addition, fungi cause serious losses in crop quality and yield. The potential for the presence of fungal toxins in commodity crops also adversely effects their value in the international marketplace. Our research is focused on species of Fusarium, which are economically important pathogens of grain crops and produce a variety of toxins such as fumonisins and deoxynivalenol. Regulatory advisories for deoxynivalenol levels in crops already exist, and guidelines for fumonisins which have recently been shown to cause cancer in rats have been proposed. The best strategy to keep these toxins from entering the food supply is to prevent them from being produced in the first place. To develop safe, reliable, and efficient ways to control fungi and their toxins, we are determining how toxins are made and regulated, as well as the role they play in the ability of the fungus to infect and cause disease in crop plants.

Summary Accomplishments during Entire Project:
We have identified and studied genes that are part of the pathway for biosynthesis and regulation of toxins in several Fusarium species. Genetically engineered strains of these fungi have been developed, and we have conducted field tests with them to rigorously establish the role toxins play in the ability of these fungi to cause Fusarium Head Blight and corn ear rot. It has been determined that fumonisins are not distributed uniformly in corn. Rather, the toxins are greatly concentrated in kernels showing disease symptoms and therefore significant lowering of fumonisin levels in corn through seed sorting is possible. The environmental distribution of strains of Fusarium verticilioides and their ability to make toxins in corn has been determined. Several closely related species of Fusarium with different host ranges have been examined for the ability to make several toxins. Through genetic analysis, sever genes involved in the synthesis of fumonisins have been identified and it has been established that they are loceated near each other along the largest chromosome of the fungus. One of these genes has been cloned and sequenced. A gene expression system which targets protein production in a very specific part of the corn kernel has been identified and is being developed. Because this is an area of the developing kernel through which fungi often enter the kernel, this system will be used to introduce genes that make products that inhibit fungal growth.

Summary 2000 Accomplishments:
Working toward our goal to increase corn's resistance to the accumulation of mycotoxins and its susceptibility to fungal diseases while the crop is in the field, we are developing a biotechnological tool, a gene promoter with the ability to express genes added to corn plants in a precise, tissue specific manner in kernel pedicels, a common site for infection of corn plants by mycotoxin producing fungi. With support from the Biotechnology Research and Development Corporation (BRDC), we are characterizing and refining the tissue- specific promoter discovered in our research to make it a user- friendly and versatile gene expression cassette system for plant breeders to add

novel genes to corn. In 2000, we filed a provisional patent application and submitted a manuscript which demonstrated that the promoter works to direct tissue specific expression of a foreign gene specifically within the corn kernel. The next step, the insertion and expression of a gene that provides antifungal proteins in this critical kernel tissue, could lead to development of new corn varieties that are more resistant to mycotoxin contamination.

The expression of additional genes located nearby the previously identified gene that is required for fumonisin production have been shown to be correlated with fumonisin production. Studies to identify the functions of these genes in fumonisin biosynthesis are nearing completion and will be reported in early 2001.

Projected Research Accomplishments During Next 3 Years:
Complete the DNA sequence of the area where the known fumonisin synthesis gene(s) are located and identify potential genes and determine potential functions by homology with public data bases. Study infection of corn plants with reporter gene constructs designed to determine when and where in the infection process fumonisin biosynthesis is induced will be completed and reported. Complete studies that determine how fumonisins are regulated in planta and field studies that determine potential for biological control of fumonisins with toxin non-producing strains will be reported.

Technology Transfer:
Information on the distribution of fumonisins in corn has been transferred to the corn processing industry. Knowledge on the biosynthesis of toxins has been transfered to the seed industry. Molecular tools developed to study the role of toxins in nature have been provided to researchers world wide. Information regarding levels of fumonisins in corn has been provided to regulatory agencies. We participated in the design and provided the methodology to purify >1000 g. of fumonisin B1 and developed criteria that established the purity of the materials used in a recently completed long-term toxicology study for fumonisin B1. This study found fumonisins are carcinogens and will provide data for a risk assessment of human fumonisin exposure and provided background data for regulatory agencies in proposing guidelines for acceptable levels for fumonisins in corn.

PUBLICATIONS:

Bai, G.-H. Inheritance of resistance to Fusarium graminearum in wheat. Theo. Appl. Gene. 2000. 100:1-8.

Desjardins, A.E., Manandhar, H.K., Plattner, R.D., Manandhar, G.G., Poling, S.P., Maragos, C.M. *Fusarium* species from Nepalese rice and production of mycotoxins and gibberellic acid by selected species. Appl. Environ. Microbiol. 2000. 66:1020-1025.

Desjardins, A.E., Plattner, R.D., Gordon, T.R. *Gibberella fujikuroi* mating population A and Fusarium subglutinans from teosinte species and maize from Mexico and Central America. Mycol. Res. 2000. 104:865-872.

Muhitch, M.J., McCormick, S.P., Alexander, N.J., Hohn, T.M. Transgenic expression of the TRI010 or PDR5 gene increases resistance of tobacco to the phytotoxic effects of the trichothecene 4,15-diacetoxyscirpenol. Plant Sci. 2000. 157:201-207.

12.4

CRIS Title: Control of *Fusarium graminearum* Mycotoxins in Wheat, Barley, and Corn
CRIS: 3620-42000-021
Scientists: Desjardins AE, Alexander NJ, McCormick SP, Proctor RH, Plattner RD
Location: Mycotoxin Research Unit, NCAUR, Peoria, IL
Contact: 309-681-6379 (P); 309-681-6689 (F); desjarae@mail.ncaur.usda.gov

Summary Project Aims:
Fusarium Head Scab is a disease of wheat and barley that has severe economic impact on U.S. agriculture. It is caused by invasion of the plants by a fungus (*Fusarium graminearum*) while the crop is growing in the field. This causes serious losses in crop quality and yield. To make matters worse, the fungus makes toxins, primarily deoxynivalenol (DON), that are harmful to humans and animals that eat toxin contaminated food or feed. The potential for the presence of fungal toxins causes extra expenses to be incurred by the grain industry for testing to assure that wheat and barley are safe for human and animal consumption and threatens the competitiveness of U.S. agriculture in the world market. The best strategy to keep these toxins from entering the food supply is to prevent them from being produced in the first place. To develop safe, reliable, and efficient ways to eliminate or minimize the ability of this fungus to infect wheat and barley, we are determining how toxins are made and regulated, as well as the role they play in the ability of the fungus to infect crop plants and cause disease.

Summary Accomplishments during Entire Project:
We have identified a number of genes that are essential for synthesis and regulation of DON and related toxins in several *Fusarium* species. Field tests using genetically engineered strains of *Fusarium graminearum* that were altered to no longer be able to make DON have shown that toxin plays an important role in the ability of the fungus to cause wheat head scab and corn ear rot. This information suggests that practices that hamper the ability of the fungus to make toxin should make the disease less severe. Toward that end, we have identified genes that allow the fungus to be resistant to its own toxins. Disruption of one of these resistance genes, TRI101, resulted in production of less phytotoxic trichothecenes and in reduced virulence on wheat heads in greenhouse tests. We have also developed bioassays using yeast and the green algae Chlamydomonas to facilitate testing of trichothecene toxicity. Putative toxin resistance genes have been expressed in plants for tests of toxin resistance, disease symptoms, and toxin levels in plants following infection with *F. graminearum*. These genes have been expressed in plants, and we are testing if they make plants resistant to the effects of DON and, therefore, lower blight symptoms and toxin levels in the plants.

Summary 2000 Accomplishments:
Progress in understanding biosynthesis and regulation of DON by *F. graminearum* has been hampered by the inability of this fungus to produce DON in liquid culture media in the laboratory. To solve this problem, we have added an extra copy of the trichothecene synthase gene promotor to produce strains of F. graminearum that consistently produce DON in liquid culture media. These strains are invaluable tools for determining the DON biosynthetic pathway, identifying DON biosynthetic genes, producing cDNA libraries, and identifying DON resistance genes.

Projected Research Accomplishments During Next 3 Years:
During the next year, we will continue to characterize genes involved in plant-fungal interactions and in toxin biosynthesis. The Chlamydomonas and yeast systems we have developed will be used to identify new toxin resistance genes from wheat, from *Fusarium*, and from other fungal sources. We will continue tests with transgenic plants that contain two previously identified toxin-resistance genes to determine if they are effective in making the plants more resistant to DON. We will identify and disrupt genes that are essential for sexual spore production in *F. graminearum* and investigate the importance of sexual spores in causing wheat head scab.

Technology Transfer:
Knowledge developed by our research concerning the role of toxins in Fusarium Head Blight has been transferred to researchers and wheat breeders and has impacted the direction of research to find effective ways to manage the disease and develop more resistant new varieties of wheat and barley. Molecular engineered tools to study the disease and infection process and measure plant resistance have been transferred to other researchers and the seed industry. Potential resistance genes for DON sensitivity have been transferred to other ARS laboratories and to the seed industry to develop transgenic wheat and barley varieties that are more resistant to Fusarium Head Blight.

PUBLICATIONS:

Chen, L., McCormick, S.P., Hohn, T.M. Altered regulation of 15-acetyldeoxynivalenol production in *Fusarium graminearum*. Appl. Environ. Microbiol. 2000. 66(5):2062-2065.

Desjardins, A.E., Bai, G.-H., Plattner, R.D., Proctor, R.H. Analysis of aberrant virulence of *Gibberella zeae* following transformation-mediated complementation of a trichothecene-deficient (*Tri5*) mutant. Microbiology 2000. 146:2059-2068.

12.6

CRIS Title: Detection, Identification, and Surveillance of Mycotoxins in Cereals and Other Foods
CRIS: 3620-42000-023
Scientists: Maragos CM, Dombrink-Kurtzman MA, Plattner RD, Vesonder RF
Location: Mycotoxin Research Unit, NCAUR, Peoria, IL
Contact: 309-681-6266 (P); 309-681-6267 (F); maragocm@mail.ncaur.usda.gov

Summary Project Aims:
Mycotoxins, naturally occurring toxins produced by fungi, are frequent contaminants of commodities important to U. S. Agriculture. Mycotoxin contamination must be controlled for three reasons: the direct economic losses resulting from lower commodity quality (this includes international trade issues), the indirect economic losses occurring from low-level exposure of domestic animals to these toxins, and the potential impact of such toxins upon human health. To ensure adequate assessment of mycotoxin levels requires the development of rapid and reliable analytical methods for the detection and quantitation of these toxins in grains, feeds, and foods. Development of such technologies also supports the research of other scientists seeking to generate plant resistance in order to reduce the occurrence of these toxins in the field. This unit is focused on developing new technologies for detection of mycotoxins, using antibody-based and contemporary analytical instrumentation. Through the development of such technologies and their transfer outside of ARS this unit contributes significantly to resolving the issues of mycotoxin analysis.

Summary Accomplishments during Entire Project:
Major accomplishments of this unit have been the development of a variety of analytical methods that have been transferred to our customers and, secondly, elucidating the fate of mycotoxins during processing of commodities. Antibodies, the essential components of immunoassay kits for screening for mycotoxin contamination, have been developed for various mycotoxins and have been distributed to several kit manufacturers. Antibodies have also been distributed to researchers outside ARS who are attempting to determine the extent of current mycotoxin problems. Biosensors have been developed for two of the mycotoxins, which may, in the future, reduce the expense and the analytical skill required to perform these tests. Capillary electrophoresis, an analytical technology which separates components of a mixture based upon electrical charge, has been developed as an alternative to traditional high performance liquid chromatography techniques for determination of mycotoxins. The effects of processing upon the fate and distribution of mycotoxins have provided insights into possible means for detoxification of commodities. In particular, the fate of fumonisins during dry-milling of corn, the effects of nixtamalization upon fumonisin levels in corn, and the effects of ozone upon deoxynivalenol in wheat. Lastly, bioassays that have been developed that have provided viable alternatives to whole animal toxicity testing.

Summary 2000 Accomplishments:
Commercial assays for deoxynivalenol (DON) were inadequate forscreening of this mycotoxin in human food, as described by a recentGAO report (GAO/RCED-99-59). At the National Center for AgriculturalUtilization Research (Peoria, IL), three monoclonal antibodies for DON were produced and enzyme linked immunosorbent assays (ELISAs) based upon them were developed. The assays,

which are the most sensitive monoclonal antibody-based immunoassays yet reported for DON, were tested with wheat samples and a Cooperative Research and Development Agreement (CRADA) is being negotiated with a mycotoxin kit manufacturer.

Projected Research Accomplishments During Next 3 Years:
Easy to use assays for the detection of fumonisins in maize, based upon the principle of fluorescence polarization (FP) will be developed. Assays will be developed for detection of DON in wheat. The mycotoxins patulin and moniliformin will be produced to permit synthesis of novel immunogens with the ultimate goal of developing antibodies for these toxins. Genetic probes will be developed for mycotoxin-producing fungi, the probes will be adapted to formats for use in presumptive tests for mycotoxins, and will be compared to standard methods to evaluate their efficacy.

Technology Transfer:
None

PUBLICATIONS:

Desjardins, A.E., Manandhar, G., Plattner, R.D., Maragos, C.M., Shrestha, K., McCormick, S.P.. Occurrence of *Fusarium* species and mycotoxins in Nepalese maize and wheat and the effect of traditional processing methods on mycotoxin levels. J. Agric. Food Chem. 2000. 48(4):1377-1383.

Hjelle, J.T., Miller-Hjelle, M.A., Nowak, D.M., Dombrink-Kurtzman, M.A., Peterson, S.W.. Polycystic kidney disease, fungi, and bacterial endotoxin: shifting paradigms involving infection and diet. Rev. Med. Microbiol. 2000. 11:23-35.

Vesonder, R.F., Wu, W., Weisleder, D., Gordon, S.H., Krick, T., Xie, W., Abbas, H.K., McAlpin, C.E. Toxigenic strains of *Fusarium moniliforme* and *Fusarium proliferatum* isolated from dairy cattle feed produce fumonisins, moniliformin and a new C21H38N2O6 metabolite phytotoxic to *Lemna minor* L. J. Nat. Toxins 2000: 9:03-112.

12.8

CRIS Title: Critical Control Points in Corn Resistance/Susceptibility to *Aspergillus flavus* and Aflatoxin
CRIS: 3620-42000-020
Scientists: Wicklow DT, Gardner HW
Location: Mycotoxin Research Unit, NCAUR, Peoria, IL
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Summary Project Aims:
In the Midwestern corn belt, the bulk of the U.S. corn crop is at risk during sporadic outbreaks of aflatoxin contamination of preharvest corn (*Zea mays* L.). Aflatoxin is a metabolite produced by the fungus *Aspergillus flavus*. The research investigates corn resistance factors that impact kernel susceptibility to *A. flavus* infection, sporulation, and aflatoxin, examines the role of fungal competition as a confounding variable in corn variety trials for aflatoxin resistance, and discovers novel metabolites with antifungal activity against *Aspergillus* and *Fusarium*.

Summary Accomplishments during Entire Project:
The present emphasis of research to control *A. flavus* and aflatoxin in preharvest corn involves the identification of relevant resistance factors in corn or biologically active proteins which may be introduced through genetic engineering. The following accomplishments have greatly improved our knowledge of corn resistance factors suggesting novel strategies for directly or indirectly controlling *A. flavus* and aflatoxin. (History/continuity: Accomplishments from CWU 3620-42000-015, terminated Sept., 1999). (1) The seed coat is a simple natural barrier to fungal infection; and we discovered that some parents of commercial corn varieties are susceptible to seed coat tearing, thus providing an entry point for *A. flavus* infection of the germ where aflatoxin is produced. This result provides corn breeders with a major all-or-nothing aflatoxin resistance trait which should have a substantial impact in reducing aflatoxin contamination of the grain at harvest. (2) The development of a method for testing compounds against aflatoxin-producing fungi which can be adapated to work with all types of compounds, including volatiles and insoluble sterols and which does so with a minimum amount of test compound and waste solvent production. (3) The discovery of new groups of naturally-occurring compoundswhich can significantly decrease the production of aflatoxin in most of the strains of *A. flavus* and *A. parasiticus* tested, including anthocyanins, carotenoids, benzoxazolinones, and D- talose, and interactions between sugars in corn which can significantly decrease the production of aflatoxin in most of the strains of *A. flavus* and *A. parasiticus* tested. (4) The discovery of a cholesterol-lowering oil in a corn milling byproduct which could have a positive impact on human health as a food additive in commercially-produced nutraceutical foods. (5) The demonstration that several pathogenesis-related proteins in maturing corn kernels are associated with *A. flavus* milk-stage infection. (6) Progress was made toward understanding the control of *Aspergillus* sporulation and how plant-fungal "cross- talk" operates through hydroxy and hydroperoxy fatty acid signals. (7) *Aspergillus flavus* strains isolated from corn showed much greater sensitivity to beta-carotene inhibition of aflatoxin B1 than several *A. flavus* strains isolated from raw peanuts. These results suggest that in fields where corn is rotated with peanuts, the grain is more likely to become contaminated with higher levels of aflatoxin. (8) We developed a molecular probe that is targeted to a DNA sequence in *A. flavus*. The probe is being used to match

strains of *A. flavus* belonging to the same genetic population in corn fields and can also be used to identify *Aspergillus* strains used in traditional oriental food fermentations (i.e., soy sauce, miso, sake) and *Aspergillus* strains isolated from at-risk patients with clinical symptoms of aspergillosis. (9) Yeasts from preharvest corn were identified, quantified, and evaluated for 'yeast killer factor' and interference with *A. flavus*. The majority of the yeasts were *Candida guillermondii*, *Candida zeylanoides*, *Debaryomyces hansenii*, and *Candida shehatae*. These results offer the potential use of antagonistic 'killer yeasts' as biocontrol agents in preharvest corn where the yeasts are also attractive to sap beetle vectors of *A. flavus*. (10) Naturally occurring fungal parasites of *A. flavus* sclerotia buried in corn fields were shown to produce novel antifungal metabolites of particular interest to the fields of agriculture and medicine.

Summary 2000 Accomplishments:
The aflatoxin in harvested grain is found concentrated at high levels in corn kernels showing a bright greenish-yellow fluorescence (BGYF) when examined under a black light, and our research seeks to eliminate these BGYF kernels through conventional breeding. An ARS scientist in Peoria, IL, in collaboration with a seed-producing company, confirmed the parental contribution to corn varietal response for numbers of BGYF kernels in a 2-year study. The results show that the frequency of BGYF kernels in experimental crosses is under the genetic control of the parents which have been classified as 'good parents' or 'bad parents'. The observations confirmed that improved corn varietal resistance to aflatoxin can be achieved by eliminating aflatoxin-susceptible BGYF kernels through a conventional breeding program.

Cereal brans are a low-value milling byproduct with limited dietary uses; new healthful properties would increase their nutritional and economic value. In collaboration with a Japanese scientist, oil extracts of corn, rye, and wheat were evaluated in an animal cell assay for inhibition of tumors resulting from the activation of Epstein-Barr virus (EBV) by certain tumor-promoting compounds. Some sterols and related compounds have previously been reported to show this activity. Results showed that sterol ferulates from rye bran, which are also present in corn and wheat, were potent inhibitors of EBV activation by a phorbol ester. Confirmation of these results by others would provide an additional reason for inclusion of corn fiber and cereal brans with related compounds in the diet.

Corn fiber oil has been patented by USDA-ARS researchers for use in lowering dietary cholesterol uptake in humans but commercialization requires a cheap source of oil with high levels of active ingredients, especially sterol ferulates. Because sporulation of *Aspergillus* often correlates with aflatoxin production, we examined *Aspergillus* sporulation factors (hydroxy fatty acids) in cooperation with Texas A&M (N. P. Keller). To accomplish these goals, strains of *Aspergillus* with a gene deletion of a delta-12 desaturase was developed at Texas A&M, and we examined the fatty acid composition as well as the sporulation factors in these strains. We found that the delta-12 desaturase deletion strain greatly modified the fatty acid composition of the fungus to produce a much elevated level of oleic acid lipids, and additionally produced elevated levels of sporulation factors of the monounsaturated type, probably accounting for their ability to sporulate. This information may be of use in designing control measures for *A. flavus*.

Lipoxygenases are often involved in fungal resistance. A corn lipoxygenase responsive to *Aspergillus* infection was developed at Texas A&M, and we characterized the product composition of this enzyme. It was found that an *Aspergillus* responsive corn lipoxygenase oxidized linoleic acid to a 9(S)-hydroperoxide, a substance known to prolong aflatoxin production by *Aspergillus*. In this work, a better understanding of how oxidized fatty acids serve as signals for plant/fungal "cross-talk pointing to possible targets for genetic modification to ameliorate aflatoxin production.

Aspergillus flavus populations in Midwestern crop fields may include a majority of individuals that produce no aflatoxin and thus could function naturally in suppressing the severity of aflatoxin outbreaks. The ability of non-aflatoxin producing isolates of *Aspergillus flavus* to interfere with aflatoxin producing isolates was evaluated by introducing varying numbers of each competitor into ear wounds. The results show that the non-aflatoxin producing isolates are largely ineffective in suppressing aflatoxin contamination unless they constitute >80% of the initial mixed population or become the initial sole colonists of kernel wounds, thereby excluding later-arriving aflatoxin-producers. The natural suppression of aflatoxin in Midwestern corn can occur when individuals from the non-aflatoxin producing population colonize ear wounds and infect individual susceptible kernels in advance of the aflatoxin producing population. An *in vitro* method was also used to determine the effect on aflatoxin production of inoculum containing varying proportions of spores from a non-aflatoxin producing strain of *Aspergillus flavus* and an aflatoxin-producing strain. Tests with several strains from each group showed that all strain combinations resulted some toxin inhibition, but the amount depended strongly on the two strains involved and the proportion of spores from each strain. The average inhibition of aflatoxin B1 for different strains ranged from 9% to 86%. These results generally confirmed that natural strain interactions can result in lower aflatoxin levels. In regions of the U.S. where corn is rotated with other aflatoxin-susceptible crops such as cotton or peanuts, ARS scientists are attempting to identify strongly biocompetitive, non-aflatoxin producing strains of *Aspergillus flavus* for biocontrol applications. DNA fingerprinting (RFLP) of non-aflatoxin producing *A. flavus* isolates from 40 farms in eastern Iowa has identified genotypes common to numerous farms. These prevalent A. flavus genotypes may prove effective biocompetitive strains in crop fields where corn is a rotation crop.

Projected Research Accomplishments During Next 3 Years:
Identify extracts of corn lines that are active against *A. flavus* and identify active compounds. Determine the effect of different types of corn protein on fat utilization for aflatoxin production. Identify the volatile component(s) in corn carotenoids which are involved in aflatoxin inhibition. Screen corn lines for levels of carotenoid aflatoxin inhibitors in germ at different growth stages, determine effect of carotene precursors. Identify the active component(s) in the breakdown products of linolenic acid (a common fatty acid) that causes inhibition of growth. Begin identification of active fraction components from corn lines, correlate activity in corn lines related to those with compound-based resistance. Identify corn varieties that can be used to improve aflatoxin resistance of corn by reducing the number of BGYF kernels through conventional breeding. Complete identification of active fraction components from corn lines and screen corn lines for levels of active components. Characterize the behaviors of plant and *Aspergillus* enzymes that produce hydro(per)oxy fatty acids as signals of sporulation so that methods can be developed to control sporulation and aflatoxin production. Cooperation with C. Hou, NCAUR, involves identification of various oxidized fatty acids produced by microbial fermentation. Define the role of intraspecific

fungal competition in naturally restricting *A. flavus* growth and aflatoxin in corn grown in the central U.S. Corn Belt. National Science Foundation sponsored research will discover novel antifungal metabolites from mycoparasites of *A. flavus* sclerotia or the persistent sporulating structures of wood decay fungi in collaboration with a university scientist.

Technology Transfer:
ARS will continue to provide materials for chemical studies by a cooperating university scientist as required by a sponsoring company through an agreement with the USDA sponsored Biotechnology Research and Development Corporation (BRDC), Peoria, IL. At the 41st Annual Corn Dry Milling Conference (June 2-3, 2000) sponsored by NCAUR and the North American Millers Association, a report was given indicating that elimination of aflatoxin contaminated BGYF kernels through conventional corn breeding could represent a major step toward elimination of aflatoxin contamination of corn prior to harvest. Negotiations concerning U.S. Patent 5,843,499 issued in 1998 to Norton et al (1998) are currently being conducted with a major U.S. food company.

PUBLICATIONS:

Bartelt, R.J., Wicklow, D.T. Volatiles from Fusarium verticillioides (Sacc.) Nirenb. and their attractiveness to nitidulid beetles. J. Agric. Food Chem. 1999. 47(6):2447-2454.

Burow, G.B., Gardner, H.W., Keller, N.P. A peanut seed lipoxygenase responsive to *Aspergillus* colonization. Plant Mol. Biol. 2000. 42(5):689-701.

Gardner, H.W., Hou, C.T. All (S) stereoconfiguration of 7,10-dihydroxy-8(E)- octadecenoic acid from bioconversion of oleic acid by Pseudomonas aeruginosa. J. Am. Oil Chem. Soc. 1999. 76(10): 1151-1156.

Gordon, S.H., Jones, R.W., McClelland, J.F., Wicklow, D.T., Greene, R.V. Transient infrared spectroscopy for detection of toxigenic fungi in corn: Potential for on-line evaluation. J. Agric. Food Chem. 1999. 47(12):5267-5272.

Ji, C., Norton, R.A., Wicklow, D.T., Dowd, P.F. Isoform patterns of chitinase and beta-1,3-glucanase in maturing corn kernels (*Zea mays* L.) associated with *Aspergillus flavus* milk stage infection. J. Agric. Food Chem. 2000. 48(2):507-511.

Kim, H., Gardner, H.W., Hou, C.T. 10(S)-Hydroxy-8(E)-octadecenoic acid, an intermediate in the conversion of oleic acid to 7,10-dihydroxy-8(E)-octadecenoic acid. J. Am. Oil Chem. Soc. 2000. 77(1):95-99.

Vesonder, R.F., Wu, W., Weisleder, D., Gordon, S.H., Krick, T., Xie, W., Abbas, H.K., McAlpin, C.E. Toxigenic strains of *Fusarium moniliforme* and *Fusarium proliferatum* isolated from dairy cattle feed produce fumonisins, moniliformin and a new C21H38N2O6 metabolite phytotoxic to *Lemna minor* L. J. Nat. Toxins 2000. 9(2):103-112.

Whyte, A.C., Joshi, B.K., Gloer, J.B., Wicklow, D.T., Dowd, P.F. New cyclic peptide and bis-indolyl benzenoid metabolites from the sclerotia of *Aspergillus sclerotiorum*. J. Nat. Prod. 2000. 63(7):1006-1009.

Wicklow, D.T. Influence of *Aspergillus flavus* strains on aflatoxin and bright greenish yellow fluorescence of corn kernels. Plant Dis. 2000. 83(12):1146-1148.

PROCEEDINGS/ABSTRACTS:

Wicklow, D.T. Aflatoxin and bright greenish-yellow fluorescence of corn kernels inoculated with Aspergillus flavus genotypes (RFLP) from a field in Illinois. Kumagai, S., editor. Matsumoto Printing Co., Tokyo, Japan. Mycotoxin Comtamination: Health Risk and Prevention Project. Proceedings International Symposium on Mycotoxicology. Chiba, Japan, Sept. 9-10, 1999. p. 246-251.

Wicklow, D.T., Dowd, P.F., Gloer, J.B. Chaetomium mycotoxins with antiinsectan or antifungal activity. Kumagai, S., editor. Matsumoto Printing Co., Tokyo, Japan. Mycotoxin Contamination: Health Risk and Prevention Project. Proceedings International Symposium on Mycotoxicology. Chiba, Japan, Sept. 9-10, 1999. p. 267-271.

Norton, R.A. Transmission of aflatoxin B1 inhibition in mycelia of *A. flavus* and other inhibitor studies. Aflatoxin Elimination Workshop. Atlanta, GA. Oct. 20-22, 1999. Abstract p. 42.

Norton, R.A. Inhibition of aflatoxin production by *A. flavus* when it invades non-inhibited media from inhibited media. NC-213 Regional Project Meeting Progress Report. Kansas City, MO. Feb. 16-18, 2000. Abstract p. 10.

CRIS Title: Biochemical, Physical, Microbiological Management for Control of Mycotoxin Contamination of Peanuts
CRIS: 6604-42000-006
Scientists: Dorner JW, Horn BW
Location: National Peanut Research Laboratory, Dawson, GA
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Summary Project Aims:
Mycotoxin contamination of crops and, particularly, aflatoxin contamination of peanuts is an important food safety issue as well as a major economic problem of the U. S. peanut industry. Aflatoxin contamination occurs when crops are infected with toxigenic strains of *Aspergillus flavus* and *A. parasiticus* under environmental conditions conducive for their growth. Two research approaches are being pursued to reduce and/or prevent aflatoxin contamination. The first is biological control, whereby non-toxigenic strains of the fungi that are responsible for contamination are applied to fields to competitively exclude the naturally present, toxigenic strains. The second is the development of a peanut cultivar that offers enhanced resistance to contamination with aflatoxin.

Summary Accomplishments During Entire Project:
Consistently reproducible reductions in preharvest aflatoxin contamination of peanuts of 70-90% have been achieved through application of nontoxigenic strains of *Aspergillus flavus* and *A. parasiticus* to peanut soils. It has also been demonstrated that treating the soil with the nontoxigenic strains has a carryover effect of protecting peanuts from aflatoxin contamination in storage. A multi-year study in which corn was grown in crop rotation with peanuts showed that applying the nontoxigenic strains to corn plots also resulted in significantly reduced aflatoxin contamination of corn. Characterization of soil populations of *A. flavus* and *A. parasiticus* along a transect extending from Virginia to New Mexico showed that soil densities of highly toxigenic strains of these fungi were quite high in certain peanut-growing regions. Such regions would benefit most from biological control measures taken to reduce the densities of these dangerous strains. Cyclopiazonic acid (CPA) is another mycotoxin produced by *A. flavus* that has been shown to be a contaminant of peanuts and corn. The competitive, nontoxigenic strains of *A. flavus* and *A. parasiticus* that have produced reductions in aflatoxin contamination have also been shown to reduce contamination of peanuts with CPA. In this regard, improved analytical methodology for CPA was developed in collaboration with Dr. F. S. Chu at the University of Wisconsin. CPA analysis has proven difficult over the years, primarily because the cleanup step prior to the quantitation of CPA in extracts produces poor recovery poor sensitivity, and poor reproducibility. The collaboration resulted in the development of an immunoaffinity column to be used for the cleanup of extracts prior to quantitation. Use of the immunoaffinity column produces highly purified extracts giving great improvements in recovery, sensitivity, and reproducibility, which greatly facilitates the analysis of commodities for CPA.

Summary 2000 Accomplishments:
Research was conducted to determine effects of field application of aflatoxin biocontrol organisms on aflatoxin contamination of peanuts stored in bulk warehouses. Peanuts from soil treated with nontoxigenic strains of *Aspergillus flavus* and *A. parasiticus* were placed in storage along with non-treated peanuts in a mini-warehouse at the National Peanut Research Laboratory and were subjected to storage conditions favoring aflatoxin contamination. Aflatoxin contamination occurring during

storage was reduced by 96.7% comparing treated and untreated peanuts. The impact of this accomplishment is that implementation of the biocontrol treatment during peanut production could significantly reduce the risk of aflatoxin contamination occurring in storage.

Natural populations of aflatoxin-producing strains of *A. flavus* and *A. parasiticus* are extremely diverse genetically; therefore, nontoxigenic biocontrol strains may differ in their effectiveness according to the genetic composition of natural populations. Research showed that the composition of the population has little effect on the capacity of nontoxigenic strains to inhibit aflatoxin production. Hence, it may not be necessary to characterize native populations in a field before application of biocontrol strains.

A significant effort in technology transfer with regard to aflatoxin biocontrol technology resulted in a major rules change by the Peanut Administrative Committee (PAC) in the way farmers' stock (FS) peanuts are marketed. The PAC administers the USDA peanut marketing agreement, and by definition, FS lots that have peanuts with visible *A. flavus* are classified as Segregation 3 and cannot be used in the edible market. The rules change adopted by PAC would not require peanuts that are treated with the biocontrol strains in the first year of an experimental use permit to be examined for visible *A. flavus*, but they would, by definition, be classified as Segregation 1 peanuts. This would allow those peanuts to be marketed in the edible trade, thus, removing a significant barrier to implementation of the biocontrol technology.

Projected Research Accomplishments During Next 3 Years:
During 2001 an experimental use permit (EUP) will be obtained for the treatment of peanuts with nontoxigenic strains of *A. flavus* and *A. parasiticus* for the purpose of biological control of aflatoxin contamination. Large-scale studies will begin under the EUP to gather data necessary to obtain full registration of the aflatoxin biocontrol product. Scale-up for production of large quantities of biocontrol inoculum will be achieved. The effect of environmental variables on the stability of aflatoxin production by native *A. flavus* populations will be determined. In 2002, a unique laboratory assay for peanut seed infection by aflatoxigenic fungi will have been developed. In 2003, new and potentially more effective fungal strains will be identified for biocontrol of aflatoxin contamination.

Technology Transfer:
A patent for the biological control of aflatoxin and cyclopiazonic acid contamination of crops has been issued. Numerous activities have been undertaken to transfer the technology to the U. S. peanut industry. Complete transfer of this technology to peanut producers hinges upon registration of the product as a biopesticide by EPA. That is the major constraint to the adoption of the technology.
Dorner, J. W., Horn, B. W., and Cole, R. J. 2000. Biological control of aflatoxin and cyclopiazonic acid contamination of crops using non-toxigenic strains of *Aspergillus*. U.S. patent 6,027,724.

PUBLICATIONS:

Cole, R., Dorner, J.W. Biological control of aflatoxin and cyclopiazonic acid contamination of peanuts. In: Mycotoxin contamination: health risk and prevention project, Kumagai, S, et al (eds.), Japanese Association of Mycotoxicology, Tokyo, 2000, p. 70-73.

Horn, B.W., Greene, R.L., and Dorner, J.W. Inhibition of aflatoxin B_1 production by *Aspergillus parasiticus* using nontoxigenic strains: role of vegetative compatibility, Biological Control. 2000 17:147-154.

PROCEEDINGS/ABSTRACTS:

Dorner, J.W., Cole, R.J., Horn, B.W., Blankenship, P.D. Evaluation of *Aspergillus oryzae* and *A. sojae* as potential biological control agents for preharvest aflatoxin contamination of peanuts. 1999. Proceedings, American Peanut Research and Education Society, v. 31:Abstract p. 58.

Horn, B.W., Greene, R.L., Dorner, J.W. Effect of peanut cultivation on soil populations of *Aspergillus flavus* and *A. parasiticus*. 1999. Proceedings, American Peanut Research and Education Society, v. 31:Abstract p. 58.

Sovolev, V.S., Cole, R.J. *trans*-Resveratrol content in commercial peanuts and peanut products. 1999. Proceedings, American Peanut Research and Education Society, v. 31:Abstract p. 60.

Horn, B.W., Greene, R.L., Dorner, J.W. Vegetative compatibility in *Aspergillus parasiticus* and its effect on the inhibition of aflatoxin production by nontoxigenic strains. 2000. Inoculum v. 51:Abstract, p. 36.

Dorner, J.W. Recent advances in analytical methodology for cyclopiazonic acid, American Chemical Society, Washington, DC, August 21-23, 2000, Division of Agricultural & Food Chemistry Abstracts. Abstract # 81.

Dorner, J.W. Correlation of *Aspergillus flavus* proliferation with aflatoxin contamination in peanuts, AOAC International, Philadelphia, PA, September 10-14, 2000. Abstract # C-209, p. 76.

Dorner, J.W., Cole, R.J., and Horn, B.W. Potential of the koji molds, *Aspergillus oryzae* and *Aspergillus sojae*, as competitive fungi in biological control of aflatoxin contamination, Aflatoxin Elimination Workshop, Atlanta, GA, October 20-22, 1999. Abstract. p. 85.

Horn, B.W., Greene, R.L., and Dorner, J.W. Role of vegetative compatibility in the biological control of aflatoxigenic fungi, Aflatoxin Elimination Workshop, Atlanta, GA, October 20-22, 1999. Abstract. p. 88.

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CRIS Title: Aflatoxin Control Through Targeting Gene Cluster Governing Aflatoxin Synthesis in Corn and Cottonseed
CRIS: 6435-41420-002
Scientists: Bhatnagar D, Cleveland TE, Cary JW, Yu J, Chang PK, Ehrlich KC, Klich MA, Rajasekaran K, Brown RL
Location: Food and Feed Safety Unit, SRRC, New Orleans, LA
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Summary Project Aims:
Aflatoxins are natural poisons produced by two common fungi, Aspergillus flavus and Aspergillus parasiticus. These fungi invade crops like corn, peanuts, cotton and treenuts and produce aflatoxins, thereby rendering these crops unfit for sale under the existing regulatory guidelines. We have been studying the genetics of this important toxin biosynthetic pathway in order to understand how and why this fungus makes aflatoxins. With this knowledge, we are developing strategies to interrupt aflatoxin synthesis, thereby preventing aflatoxin contamination of crops. We have characterized the genetics of the entire aflatoxin biosynthetic pathway including the mechanism of correlation between toxin biosynthesis and fungal development in these two fungi. We are using this information to assist in the development of host-resistance against aflatoxin contamination by studying the effects of various physiological parameter, e.g. drought stress on gene expression in the toxigenic fungi. We have also acquired the ability to interrupt the machinery in the fungus required to produce these toxins, so that we can develop atoxigenic fungal strains that can be used as biocontrol agents.

Summary Accomplishments during Entire Project:
This project is the "parent project" of the following cooperative agreements involved in like research - Keller/Texas A&M University; Bennett/TulaneUniversity; Kikkoman Corporation. Studies were conducted on fungal deoxyribonucleic acid (DNA) and one region of the genome (the sum of all the genes in a cell of the organism) was identified in the fungus which was found to house all the genes encoding enzymes that catalyze the chemical reactions leading to aflatoxin formation. In addition to at least 20 genes discovered to be involved in aflatoxin production, several additional genes that may impact toxin synthesis have been identified. A gene encoding a membrane bound secretory protein was also discovered and characterized. This gene could be involved in aflatoxin secretion out of the fungal cell and, thus, would provide a target for aflatoxin inhibition. The sugar utilization pathway gene cluster (four genes) has also been localized. Characterization of these genes could lead to our understanding of how simple sugars support toxin formation. Additionally, we have defined one end of the aflatoxin pathway gene cluster. The functional aspects of master switch gene(aflR), in the same region of DNA that governs aflatoxin production, were studied in details and which could be targeted essentially to switch off aflatoxin production. We have also determined AFLR-binding sites in aflatoxin pathway genes and identified the domain of the aflatoxin biosynthesis regulatory protein that is responsible for turning on of the pathway genes. Research to disrupt genes in the aflatoxin gene cluster and convert aflatoxin producing strains to non-producing strains was developed. These disarmed aflatoxin non-producing strains could be applied to the field to out compete toxin producing strains or use in industrial applications. Also, we have developed an efficient method for the generation of large deletions within the Aspergillus parasiticus aflatoxin pathway gene cluster. aflR mutants of Aspergillus parasiticus have also been generated

that can be used to study aflatoxin production and its regulation. We have correlated the inability of aflatoxin production of food-grade A. sojae with a defect in this region of DNA (aflR gene). Through studying a non-toxigenic strain in the A. flavus group (A. sojae) used in industrial fermentations, it was discovered that the master gene in this fungal strain contained a mutation (stop codon) leading to premature termination of synthesis of the regulatory protein (aflR). Thus, with the absence of the functional regulatory protein, no induction of pathway genes was possible. Reporter genes (yielding an easy to visualize color when the genes are active) were engineered in the laboratory and introduced into the fungus for rapid assessment of aflatoxin gene expression when plants are invaded by the fungus or when the conditions are optimal in the field for toxin production. A relationship between fungal development (e.g. production of reproductory structures such as spores) and aflatoxins synthesis was established. Knowledge of fungal development and associated aflatoxin inductive mechanisms could lead to a dual strategy to destroy both the fungus reproductory (survival) and aflatoxin producing capabilities.

Summary 2000 Accomplishments:
Aflatoxins are toxic and carcinogenic compounds produced by several fungi of the Aspergillus family, primarily A. flavus and A. parasiticus, when these organisms infect crops, such as corn, cotton, peanuts, and treenuts. We have been eliminating the steps (genes) that govern toxin synthesis in these fungi to devise ways to stop the fungus from making these toxic compounds in food and feed. Based on the characterization of the aflatoxin pathway genes, we have developed "tester" strains of the toxigenic fungus to monitor the progression of aflatoxigenic fungi during invasion of corn and cotton and their ability to make toxin under various environmental conditions. These strains are being used this year to inoculate corn to study the progression of A. flavus during invasion of corn. These tester strains of the fungus will allow us to rapidly screen germplasm, both in the lab and in the field, to determine its resistance to fungal invasion and toxin formation. Additionally, an understanding of the genetics of toxin formation has also enabled us to design non-toxic strains that could be useful as biological control agents for reducing the presence of toxin producing strains on the crops.

Projected Research Accomplishments during Next 3 Years:
The complete characterization of aflatoxin genes and their regulation will not only be extremely beneficial in our understanding of how and why the toxin is produced by the fungus when it invades a crop, but will also aid in the successful completion of other projects seeking to develop non-aflatoxigenic biocompetitive fungi or to monitor crop resistance to fungal growth and aflatoxin formation. The following objectives will be accomplished over the next three years: Determine the molecular basis for aflatoxin inhibition/initiation in the fungus by plant metabolites to understand factors that are necessary for natural resistance to aflatoxin expression during fungal invasion. Continue to study the molecular regulation of aflatoxin synthesis, particularly in relationship to various physiological factors. Also, determine the evolutionary significance of aflatoxin production by various strains of the Aspergillus family of fungi. Identify factors in plants that impart resistance to field accumulation of aflatoxin, and attempt to move such desirable traits into corn/cottonseed. This will be critical to generating new varieties of corn or cottonseed that will have natural resistance to aflatoxin production and yet will not have undesirable traits that may render them unsuitable as a food source. This work will supplement the ongoing Host-Resistance CRIS (#6435-42000-012).

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Determine the molecular basis for natural non-production of aflatoxin by *A. flavus*. This will be critical to finding an ideal bio-competitor A. flavus that will not present a future source of aflatoxin contamination, thereby ensuring longterm safety and efficacy of biological control agents. This study will greatly enhance the viability and suitability of the Biological Control CRIS (#6435-42000-014).

Conduct further experiments to determine how environmental conditions (such as nitrogen/carbon sources, temperature, water activity, etc.) influence the genetic regulation of toxin synthesis. The nitrogen metabolism gene cluster, the sugar utilization gene cluster, the aflatoxin transporter gene will be characterized in details to develop additional strategies to control aflatoxin formation by the fungus.

Technology Transfer:
A Trust Fund agreement was entered into with Kikkoman Corp. (total nearly $200,000 granted to project) to investigate the stability and mechanism (of aflatoxin non-production) of A. sojae (in the A. flavus group) used in industrial fermentations in Japan and in the U.S. Unit scientists participated in two high impact, customer oriented meetings this year. At the ARS Workshop on Aflatoxin Prevention in Southern Corn meeting held at the Southern Regional Research Center (SRRC) in New Orleans, LA, technical advice from project experts from SRRC and other ARS locations was presented to attempt to solve the toxin contamination problem in Southern corn. Scientists of the unit, at meetings by invitations only, presented significant research results at two of the premier scientific meetings, the Fungal Genetics Meeting in Asiloman, CA and the Gordon Research Conference on mycotoxins and phycotoxins. Project scientists participated in the Aflatoxin Elimination Workshop (St. Louis, MO), presented their research and interacted (obtained customer input) with members of the American Corn Millers Federation, National Corn Growers Association, Corn Refiners Association, National Cottonseed Products Association, National Cotton Council, American Peanut Council, Dry Fruits Association, Almond Board of California, California Pistachio Commission, and the Walnut Marketing Board. Scientists on this project have six cooperative agreements with universities whose research is highly dependent upon cooperative interactions with ARS. These include Tulane University, Xavier University, University of Wisconsin, Texas A&M University (two agreements), and North Carolina State University. These cooperative agreements are supported by additional appropriated funds ($93,000) which are utilized to fund research of university cooperators associated with the project; these cooperators provide expertise (e.g., production of monoclonal antibody probes; production of reporter gene-containing strains of A. flavus) which would otherwise be unavailable to ARS for carrying out various aspects of the project. This additional funding is highly supported by a large number of industries with great interest in the technology, which documents the potentially large benefits which could be derived from the use of aflatoxin control products developed by project scientists and cooperators.

PUBLICATIONS:

Yu, J., Chang, P.-K., Bhatnagar, D., Cleveland, T. E. Genes encoding cytochrome P450 and monooxygenase enzymes define one end of the aflatoxin pathway gene cluster in Aspergillus parasiticus. Applied Microbiology and Biotechnology. 2000. v. 53. p. 583-590.

Chang, P.-K., Yu, J., Bhatnagar, D., Cleveland, T. E. Characterization of the Aspergillus parasiticus major nitrogen regulatory gene, areA. Biochimica Et Biophysica Acta. 2000. v. 1491. p. 263-266.

Cary, J.W., Ehrlich, K.E., Wright, M.S., Chang, P.-K., Bhatnagar, D. Generation of aflR disruption mutants of Aspergillus parasiticus. Applied Microbiology and Biotechnology. 2000. v. 53. p. 680-684.

Cary, J. W., Linz, J. E., Bhatnagar, D. Aflatoxins: Biological significance and regulation of biosynthesis. Cary, J. W., Linz, J. E., Bhatnagar, D., editors. Technomic Publishing Co., Lancaster, PA. Microbial Foodborne Diseases: Mechanisms of Pathogenesis and Toxin Synthesis. 2000. p. 317-361.

Cary, J. W., Chang, P.-K., Bhatnagar, D. Clustering of metabolic genes in filamentous fungi. Applied Microbiology and Biotechnology. Springer-Verlag, UK. 2000. (Invited review). 11 pages.

Cary, J. W., Montalbano, B. G., Ehrlich, K. C. Promoter elements involved in the expression of the Aspergillus parasiticus aflatoxin biosynthesis pathway gene avnA. Biochimica Et Biophysica Acta. 2000. v. 1491. p. 7-12.

Cary, J. W., Montalbano, B., Ehrlich, K. C. Promoter elements involved in the expression of the Aspergillus parasiticus aflatoxin biosynthesis pathway gene avnA. Biochimica Et Biophysica Acta. 2000. v. 91377. p. 1-6.

Bhatnagar, D., Ehrlich, K. C., Chang, P.-K. Mycotoxins in agriculture. Encyclopedia of Life Sciences. McMillan Co., London. 2000. 13 pages.

Yu, J., Chang, P.-K., Bhatnagar, D., Cleveland, T.E. Genes encoding cytochrome P450 and monooxigenase enzymes define one end of the aflatoxin pathway gene cluster in Aspergillus parasiticus. Applied Microbiology and Biotechnology. 2000. v. 53. p. 583-590.

Chang, P.-K., Yu, J., Bhatnagar, D., Cleveland, T.E. Characterization of Aspergillus parasiticus major nitrogen regulatory gene, areA. Biochimica Et Biophysica Acta. 2000. v. 1491. p. 263-266.

Yu, J., Woloshuk, C.P., Bhatnagar, D.,Cleveland, T.E. Cloning and characterization of avfA and omtB genes involved in aflatoxin biosynthesis in three Aspergillus species. Gene. 2000. v. 248. p. 157-167.

12.20

PROCEEDINGS/ABSTRACTS:

Klich, M. A. Biogeography of Aspergillus Species. Inoculum. 2000. 51(3). Abstr. p. 40.

Bhatnagar, D., Yu, J., Chang, P.-K., Cleveland, T.E. Hexose transport protein is genetically linked to aflatoxin production in Aspergillus parasiticus and Aspergillus flavus. IXth Congresses of Bacteriology and Applied Microbiology and Mycology. 1999. p. 246.

Chang, P.-K., Yu, J., Ehrlich, K.C., Boue, S.M., Montalbano, B.G., Bhatnagar, D., Cleveland, T.E. Disruption of the aflatoxin biosynthetic gene adhA in Aspergillus parasiticus leads to the accumulation of 5'-hydroxyaverantin. Annual Meeting of the American Society for Microbiology. Los Angeles, CA. 2000. p. 507.

CRIS Title: Aflatoxin Control Through Addition of Enhancement of Antifungal Genes in Corn and Cotton.
CRIS: 6435-42000-012
Scientists: Cleveland TE, Cary JW, Rajasekaran K, Brown RL, Klich MA, Jacks TJ, Yu J
Location: Food and Feed Safety Unit, SRRC, New Orleans, LA
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Summary Project Aims:
Drought stress at critical times during kernel or seed development and/or insect injury of crops can lead to susceptibility to attack by the aflatoxin-producing fungus, Aspergillus flavus. Aflatoxin detected at levels of 20 parts per billion (ppb) (established by the FDA) or above can make the crop unsalable. Aflatoxin is considered to be toxic to livestock being fed contaminated grain and to be a potent carcinogen correlated with liver cancer in certain human populations around the world. The specific goal is to develop resistant crops to fungal disease to reduce the incidence of aflatoxin contamination. Resistance related plant chemicals, proteins and genes are being identified for use in marker assisted breeding of corn and genetic engineering of cotton are being utilized to enhance resistance to A. flavus infection and aflatoxin contamination in these two crops. Development of aflatoxin control procedures will contribute to a safer food/feed supply, decrease economic losses associated with disposal of unsafe food/feed, provide environmentally friendly biologically based technology to solve the aflatoxin problem, and increase the competitive ability of U.S. growers in export markets through providing them with a wholesome, aflatoxin free crop.

Summary Accomplishments during Entire Project:
This project is the "parent project" of several cooperative agreements involving University scientists engaged in identification of resistance traits in corn (Woloshuk/Purdue University and Damaan/Louisiana State University) and identification (Tuzun/Auburn University) and insertion of resistance genes into crop plants including cotton (Chlan/University of LA at Lafayette) and peanut (Weissinger/North Carolina State University and Ozias-Akins/University of Georgia). Recently discovered corn kernel proteins and associated genes are being mapped for use as selectable markers to study the inheritance of resistance in maize to A. flavus invasion (in cooperation with the ARS-USDA Corn Host Plant Resistance Research Unit at Mississippi State University). Some of these proteins in maize kernels were shown to be in in vitro assays and were shown to be present in higher quantities maize varieties demonstrating resistance to invasion by A. flavus. Maize varieties from the germplasm collection at the International Institute of Tropical Agriculture, Nigeria, were screened for kernel resistance (using Kernel Screening Assay (KSA)); KSA's of this germplasm revealed new sources of resistance comparable to the best germplasm sources in the U.S. To identify antifungal genes for possible use in genetic engineering of plants, fungicidal properties and binding to fungal membrane components of a number of plant proteins and synthetic peptides were studied. Chemical studies demonstrated a strong correlation between chemical properties and antimicrobial potency of the peptides, this indicating possible tools to predict and optimize peptide potency. Genes encoding a peptide (D4E1), haloperoxidase (an enzyme) and a corn antifungal protein (14 Kd trypsin inhibitor) have been introduced into tobacco and cotton by genetic engineering for evaluation of the plants for resistance to fungal pests. Tobacco plants and cotton callus tissue expressing these

antifungal agents have shown efficacy in inhibiting the growth of A. flavus and other pathogenic fungi. Transgenic tobacco containing new antifungal genes was resistant to attack by the foliar pathogen, Colletotrichum. Pre-treatment of cotton bolls with fungal-derived elicitors and plant defense chemical "activators" to induce native defenses, followed by inoculation with A. flavus, resulted in lower levels of aflatoxin in the seed relative untreated bolls. The results suggested that cotton has a native capability to resist A. flavus invasion providing defense mechanisms can be induced to effective levels before fungal attack. Stress conditions which may compromise resistance, similar to those in Arizona desert-grown cotton, were mimicked in the greenhouse so that mechanisms of the stress-induced increase in natural infection by A. flavus of nectaries (natural openings by which a fungus might invade the cotton plant) and cottonseed could be studied at the biochemical level. The overall impact of this research is that the discovery of native plant resistance markers and of antifungal genes of other organisms and their mechanisms of induction could provide the technology to improve crop resistance to A. flavus (and aflatoxin). Technology could be implemented through plant breeding, genetic engineering or through induction of chemical resistance responses with certain elicitor treatments.

Summary 2000 Accomplishments:
Methods need to be developed through research to prevent aflatoxin contamination of crops before harvest, since the presence of this toxin in even very low amounts makes foods and feeds unacceptable for animal and human consumption due to its toxic and carcinogenic properties. Using a new protein analysis technique (proteomics). We have discovered a diverse family of proteins in corn kernels that probably act together to inhibit the fungus (A. flavus) that produces aflatoxin in this crop. This research led to the major accomplishment of this reporting period, which is the identification of the genes encoding these inhibitory proteins in corn. The impact of this discovery is that knowing the identity of genes encoding these inhibitors of A. flavus has led to strategies to increase resistance to aflatoxin contamination in corn through selection for the genes during breeding, or even in different crop species (e.g. cottonseed) through insertion of the genes by genetic engineering, thus reducing or eliminating this serious food safety and economic threat to U.S. grains.

Projected Research Accomplishments during Next 3 Years:
Conduct bioassays on mature cotton bolls of genetically engineered cotton in the greenhouse, expressing foreign antifungal genes, in order to determine levels of resistance in these new plant lines to invasion by A. flavus. Develop protein identification methods (proteomics), microsequencing (determination of the proteins' amino acids) and antifungal protein identification technologies for cloning of antifungal genes in U.S. and African maize germplasm. Within the second year, the goals would be to: 1) Develop rapid tests for determination of biochemical markers linked to resistance to A. flavus for use by plant breeders in marker assisted breeding of commercial corn hybrids, 2) transform cotton with identified corn genes encoding antifungal proteins, 3) assess in the greenhouse transformed cotton for new antifungal gene activities obtained from corn and other sources, and 4) begin protocols for corn transformation to up-regulate antifungal genes in commercial corn lines (tentative). During the first two years, research will also be continued to identify additional useful resistance markers and antifungal genes and their inductive mechanisms in the plant, and to determine the basis for stress induced vulnerability to A. flavus invasion of

nectaries, cotton bolls and seed. During year three, it is hoped that technologies to improve resistance to aflatoxin in cottonseed can be implemented in the field. Also, at the beginning of year three, it is hoped that a functional corn transformation system for up-regulating resistance genes active against A. flavus and/or aflatoxin will have been developed and transformed corn lines will be in the greenhouse testing phase.

Technology Transfer:
A three-way Cooperative Research and Development Agreement (CRADA) was continued between ARS, Mycogen Corporation (now Dow Chemical Co.), and University of Southwestern Louisiana to genetically engineer cotton with antifungal peptide genes for resistance to A. flavus and to introgress disease resistance trait(s) into cotton expressing insect resistance (Bt) traits. Scientists on the project have 6 cooperative agreements with universities to genetically engineer cotton and peanut and enhance resistance in corn by marker based breeding to reduce aflatoxin contamination. These include University of Louisiana at Lafayette, Louisiana State University, Purdue University, Auburn University, University of Georgia, and North Carolina State University. These cooperative agreements are supported by additional appropriated funds which are utilized to fund research of university cooperators associated with the project; these cooperators provide expertise (examples, production of monoclonal antibody probes; production of reporter gene-containing strains of A. flavus) which would otherwise be unavailable to ARS for carrying out aspects of the project. This additional funding is highly supported by a large number of industries with great interest in the technology, which documents the potentially large benefits which could be derived from the use of aflatoxin control products developed by project scientists and cooperators. Project scientists participated in the Aflatoxin Elimination Workshop Yosemite, Ca., October 25-27, 2000, which is a high impact, customer oriented meeting in order to transfer available knowledge and technology to industry, farmers, and other scientists in the area of aflatoxin prevention research and technology. The scientists presented their research and interacted (obtained customer input) with members of the Corn Refiners Association, National Peanut Foundation, Corn Millers Association, Cerestar USA, Anderson Clayton Co, National Cottonseed Products Association, National Cotton Council, Golden Peanut Co., Beatrice-Hunt Wesson, National Peanut Growers, Best Food, Georgia Peanut Commission, Dried Fruit Association, Proctor and Gamble Corporation, National Corn Growers Association, Almond Board of California, Hunt-Wesson, Inc., Paramount Farms of California, and the Walnut Marketing Board. Technologies to prevent aflatoxin on cottonseed using a genetic engineering approach to enhance host crop resistance may become available by the year 2001. However, end users will probably not be able to implement the technology until at least 2004 or 2005 because of the logistics of field testing and regulatory requirements of releasing genetically engineered plant varieties into the environment. Providing technology to enhance resistance to aflatoxin in corn through plant breeding will probably take approximately 4 to 5 more years. Implementation of resistant corn hybrids on a commercial scale may take 10 more years, because of the difficulty of incorporation of multiple resistance traits into corn together with desirable agronomic traits.

PUBLICATIONS:

Cary, J.W., Rajasekaran K., Jaynes J.M., Cleveland T.E. Transgenic expression of a gene encoding a synthetic antimicrobial peptide results in inhibition of fungal growth in vitro and in planta. Plant Science. 2000. v. 154. p. 171-181.

Rajasekaran, K., Hudspeth, R.L., Cary, J.W., Anderson, D.M., Cleveland, T.E. High-frequency stable transformation of cotton (Gossypium hirsutum L.) by particle bombardment of embryogenic cell suspension cultures. Plant Cell Reports. 2000. v. 19. p. 539-545.

Rajasekaran, K., Finley, J.W., Jacks, T.J. Genetic Engineering to improve attributes of crop plants. In "Celebration of Chemistry in the 21st Century." American Chemical Society Books. 2000. p. 1-4.

Jacks, T.J., Rajasekaran, K., Cary, J.W., Cleveland, T.E., van Pée, H.-K. Transformation of plants with a chloroperoxidase gene to enhance disease resistance. 2000. U.S. Patent (ARS Docket No. 0021.98).

Rice, D., Coruzzi, N., Anderson, D.M., Rajasekaran, K., Rangan, T.S., Yenofsky, R.L., Lotstein, R. Cotton Promoter. 2000. U.S. Patent 6,040,504.

Wright M.S., Green-McDonald D.M., Zeringue, H.J.,Jr., Bhatnagar, D., Cleveland, T.E. Effects of volatile aldehydes from Aspergillus resistant varieties of corn on Aspergillus parasiticus growth and aflatoxin biosynthesis. Toxicon. 2000. v. 38. p. 1215-1223.

Zeringue, H.J., Jr. Identification and effects of maize silk volatiles on cultures of Aspergillus flavus. Journal of Agricultural and Food Chemistry. 2000. v. 48. p. 921-925.

Zeringue, H.J., Jr., Shih, B.Y., Maskos, K., Grimm, D. Identification of the bright-greenish-yellow-fluorescence (BGY-F) compound on cotton lint associated with aflatoxin contamination in cottonseed. Phytochemistry. 1999. v. 52. p. 1391-1397.

PROCEEDINGS/ABSTRACTS:

Cleveland, T.E., Bhatnagar, D., Yu, J., Chang, P-K., Rajasekaran, K., Brown, R.L., Cary, J.W. Metabolic engineering applications for control of aflatoxin produced by Aspergillus species. Society for Industrial Microbiology Meeting. 1999.

Cleveland, T.E., Brown, R.L., Chen, Z., Cary, J.W., Rajasekaran, K., Jacks, T.J., Bhatnagar, D. Identification of antifungal genes from corn and other sources for enhancement of host plant resistance to invasion by mycotoxin-producing fungi. Fumonisin Risk Assessment Workshop. 2000.

Rajasekaran, K., Cary, J.W., Cleveland, T.E. Expression of a gene encoding a synthetic antimicrobial peptide confers fungal resistance in vitro and in planta in transgenic plants. 6th International Conference on Plant Molecular Biology. 2000.

Jacks, T.J., Rajasekaran, K., Cary, J.W., Cleveland, T.E., van Pee, H.-K. Increased disease resistance in transgenic plants expressing a bacterial nonheme chloroperoxidase gene. World Congress Biotechnology.11th International Biotechnology Symposium and Exhibition. 2000.

12.26

CRIS Title: Modification of Fungal Community Structure to Improve Food Safety
CRIS: 6435-42000-014
Scientists: Cotty PJ, Mellon JE
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Summary Project Aims: Aflatoxins are carcinogenic toxins produced by members of the Aspergillus flavus group of fungi. Contamination with aflatoxins is the most important problem associated with crop infection by the fungus Aspergillus flavus. Many countries have regulations prohibiting contamination above specified limits. In the U.S. contamination of foods and many feeds may not exceed 20 parts per billion (ppb). Agricultural products that contain excess aflatoxins have greatly reduced value. There are no methods growers can apply to reliably and economically prevent aflatoxin contamination. Growers are at the mercy of the environment; heat and drought combined with humidity as the crop reaches maturity frequently result in disastrous levels of aflatoxins. The project seeks to develop management methods from improved understanding of both the ecology of the fungi causing contamination and the characteristics of crops that favor contamination. Strains of Aspergillus flavus that do not produce aflatoxins (atoxigenic strains) are potentially useful tools for limiting contamination. By competitively excluding aflatoxin producers, atoxigenic strains reduce the aflatoxin producing potential of fungi resident in agricultural areas and thus reduce aflatoxin contamination of the crops produced. Although a conceptual framework for the use of atoxigenic strains is well developed. Knowledge of effects of agronomic practices and environmental variability on atoxigenic strain behavior is needed to develop the technology to practical use. Questions involving biopesticide registration, fungal ecology, stability and movement of fungal communities and practical manufacture and on farm use of this potentially useful biopesticide need to be addressed. In collaboration with grower organizations, farm and gin based studies on impacts of agronomic practices on contamination are combined with large commercial field tests of atoxigenic strain technology in order to develop procedures that may be useful in area-wide aflatoxin management programs. Through detailed laboratory and field studies, the project seeks improved understanding of the population biology, ecology, and physiology of the causative agents of aflatoxin contamination and of biochemical aspects of crop susceptibility and the contamination process.

Summary Accomplishments during Entire Project:
This project is the "parent project" of the following cooperative agreements involved in like research: Cardwell/International Institute of Tropical Agriculture, Benin, West Africa; Cotton Incorporated; Cotton Foundation; Antilla/Arizona Cotton Research & Protection Council; Nelson/University of Arizona. Aspergillus flavus, the causal agent of aflatoxin contamination, was found to be highly diverse genetically, morphologically, and physiologically. Natural strains of A. flavus that do not produce aflatoxins were found capable of competitively excluding aflatoxin producers and in so doing, reducing aflatoxin contamination. Field tests supported the use of atoxigenic strains in aflatoxin management and procedures for practical application of strains were developed. Procedures to produce significant quantities of atoxigenic strain material in the laboratory were developed and an experimental use permit was obtained from the Environmental Protection Agency (EPA) that permitted treatment of 1100 acres from 1996 through 1998. Test results indicated efficacy under commercial conditions and follow-up studies during 1999 and 2000

revealed some long-term influence of atoxigenic strain applications. This suggested that repetitive treatment of areas might result in a gradual, area-wide reduction in the vulnerability of all crops grown in a treated area to aflatoxin contamination. In collaboration with the Interregional Research-4 project, materials were developed and submitted to the EPA to support both expansion of the ARS Experimental Use Permit (EUP) for atoxigenic strain Aspergillus flavus AF36 to 20,000 acres per year (for 1999 and 2000) and a full registration for the state of Arizona requested by the Arizona cotton producers. The application for the EUP was successful. The section 3 application is still pending further material. Diverse data on fungal communities in areas treated with atoxigenic strains and in areas not treated were generated and provided to the EPA for consideration. Procedures to produce atoxigenic strain inoculum were characterized and optimized.

Development of techniques to scale-up production of inoculum to commercially significant levels was initiated and completion of the first step in developing scaled-up technology (completion of a 30 cu ft multi-step batch system) was completed. Techniques to quantify the incidence of atoxigenic strains in the air were developed and the impact of applications on fungi in the air was assessed. Atoxigenic strain applications reduced the amount of aflatoxin producers in the air without increasing the total quantity of fungi in the air. Aflatoxin production of the A. flavus communities in natural habitats was analyzed and compared to communities in agricultural areas. The composition and incidence of natural communities is an important aspect of materials submitted to the EPA for registration of atoxigenic strains. Communities of A. flavus resident in native Sonoran desert habitats have a lower ability to produce aflatoxins than those in agricultural fields. Debris from several plants is conducive to A. flavus growth in natural habitats.

The incidence of atoxigenic strain AF36 was assessed in natural habitats in the Sonoran desert. Media simulating compositions of cottonseed and corn were used to describe A. flavus substrate preferences. These were combined with data on A. flavus behavior during crop infection and used to describe how A. flavus makes aflatoxins during crop contamination. The fungus can use fats instead of sugars to produce both aflatoxins and biomass. However, simple sugars are preferred and proteins can stimulate increased aflatoxin biosynthesis. Unusual strains of A. flavus from West Africa were discovered. These strains produce aflatoxins and are proving useful in understanding how aflatoxin production is regulated.

Summary 2000 Accomplishments:
Natural habitats of the Sonoran desert were examined in order to quantify exposure to A. flavus and aflatoxins in natural habitats and to identify ecological niches harboring reservoirs of A. flavus. This information has value both during registration of atoxigenic strains of A. flavus as biopesticides and in developing tools for reducing aflatoxin contamination. We found Aspergillus flavus to be widely distributed in natural habitats and to occur at particularly high concentrations on plant debris and dung. Key food and shelter sources for wildlife supported high populations of A. flavus. Legume fruits were found frequently contaminated with aflatoxins. Aflatoxin contamination is typically associated with agriculture, this is the first evidence that contamination is a frequent, natural phenomenon that may impact fauna even in habitats removed from agriculture. Reservoirs of A. flavus not previously recognized exist in natural habitats and should be considered by aflatoxin management programs.

Lipoxygenase has been investigated for its potential to increase resistance against aflatoxin contamination in certain susceptible hosts of A. flavus. Researchers have sought to use biotechnology to put soybean lipoxygenases in susceptible hosts and thus, in theory, reduce susceptibility of those hosts to contamination. To improve flavor characteristics, soybean lines have been bred lacking one or all three natural soybean lipoxygenases. Susceptibility to aflatoxin contamination among soybeans varying in expressed lipoxygenase was investigated in order to test if altering soybean lipoxygenase expression changed soybean susceptibility to aflatoxin contamination. Several lines of test results suggest increased lipoxygenase expression does not increase soybean resistance to aflatoxin contamination. These results suggest that genetically engineering crops susceptible to aflatoxin contamination to express soybean lipoxygenases will not greatly decrease susceptibility to aflatoxins.

Projected Research Accomplishments during Next 3 Years:
In partnership with the Arizona Cotton Research and Protection Council, procedures that a grower run organization can use to produce quantities of atoxigenic strain inoculum that are sufficient to run an area-wide aflatoxin elimination program will be sought. Initial evaluations of the influence of agronomic practices on atoxigenic strain technology will be assessed. Improved methods to evaluate area-wide influences of treatments will be developed. Importance of cacti in the life cycles of aflatoxin producing fungi will be assessed. The distribution of highly toxigenic and atoxigenic strains of A. flavus in South Texas will be characterized. Unusual A. flavus strains will be further examined to assess ecological and etiological roles and to obtain insights into regulation of aflatoxin. Details on utilization of corn constituents by A. flavus during kernel infection and contamination will be sought. Characterize the influence of flooding and water potential on atoxigenic strain inoculum.

Develop procedures will be used to assist the Arizona cotton producers in developing a facility to produce inoculum for a statewide program directed at reducing aflatoxin contamination of cottonseed. Develop recommendations on optimal irrigation, cultivation, and application practices for atoxigenic strain use. Techniques to measure migration into and emigration from treatment areas will be developed and applied. Development and testing of spore dispersal stations will be initiated. Results of commercial-scale and field-plot tests will be used to assist the Arizona cotton growers in planning and implementing an area-wide aflatoxin elimination program based on atoxigenic strains. The roles of corn protein, starch, and fat in driving aflatoxin biosynthesis will be characterized. The sequence of nutrient utilization during corn infection and contamination will be initially assessed as part of this process. Studies on the progress of disease under different environmental conditions will be applied to better characterize the second phase of aflatoxin contamination of cottonseed in order to improve recommendation for managing late season aflatoxin increases. Seasonal shifting in aerial fungal communities will be further characterized and the influence of atoxigenic strain applications on these shifts will be assessed. Personnel in Arizona will be trained to perform evaluations of air, soil, and crop fungal communities. Develop a laboratory system for studying fungal processes in soil. Complete characterization of temporal cycles of A. flavus communities in natural Sonoran desert habitats and in agriculture and assess impact of natural reservoirs on agriculture.

Field-testing and evaluations will be used to begin the process of optimizing area-wide and long-term aspects of atoxigenic strain use. Influences of crop rotations and post-harvest debris management on long-term influences will be sought. Spore dispersal stations will be assessed as methods to supplement broadcast applications and will be utilized to study mechanisms of dispersal and product take away. Studies will be initiated to optimize cultural practices such as irrigation, cultivation, and planting date. Emphasis will be placed on elucidating ecological factors that both constrain fungal community modification and limit the stability of modified communities. Influences of climate, geographic location, and season on natural communities of Aspergillus in section flavi will be assessed and the underpinning factors dictating regular seasonal variations in A. flavus strain composition will be sought. Natural factors affecting A. flavus strain distribution will also be sought with the hope of identifying ecological niches preferentially favorable for strains with specific aflatoxin phenotypes. The importance of specific natural desert substrates and hosts to commercial crop infection will be compared and desert reservoirs of A. flavus will be characterized. The distribution and composition of A. flavus communities in south Texas will be mapped and contrasted with geographical and ecological features and with aflatoxin contamination of crops. Assays will be developed to determine phenotypic divergence among A. flavus communities in south Texas, Arizona, and the southeastern United States. Criteria for selecting strains best adapted for each region will be sought based on adaptive traits. Characterize activities of A. flavus in non-sterile agricultural soils under conditions varying in temperature and water availability.

Technology Transfer:
Technologies associated with utilizing atoxigenic strains of A. flavus to prevent aflatoxin contamination are in the process of being transferred to the Arizona cotton producing community. In the process of adapting these technologies for use by commercial entities, we are attempting to solve difficulties as they are encountered in order to speed adaptation. Technologies transferred include simple strain identification, starter culture procedures, scale-up procedures, quality control procedures, and methods to assess efficacy. A prototype facility using ARS technology for producing pilot-scale quantities of inoculum is up and functioning. The facility is owned and operated by the Arizona Cotton Research and Protection Council. The greatest obstacle to implementation currently is the pesticide registration process in which we are engaged. There are also still considerable technological obstacles to overcome and the logistic problems of developing a management program unlike any previously directed at a plant pathogen. Through consultations and seminars we have transferred management recommendations for reducing aflatoxin contamination of cottonseed and recommendations for dealing with aflatoxin contamination at the gin and oil mill levels. Consultations on management of contamination of other crops (peanuts, corn, tree-crops) also occur on a regular basis. This has included consultations with growers, corporations and state and federal agencies. We have transferred techniques to identify fungal strains and genetic groups with genetic techniques and by vegetative compatibility analysis to scientists. These techniques are currently being utilized. We have also transferred techniques and knowledge useful in studying the population biology and ecology of A. flavus.

12.30

PUBLICATIONS:

Mellon, J.E., Cotty, P.J., Dowd, M.K. Influence of lipids with and without other cottonseed reserve materials on aflatoxin B_1 production by Aspergillus flavus. Journal of Agricultural and Food Chemistry. August, 2000. v. 48(8). p. 3212-3220.

Bock, C.H., Cotty, P.J. Wheat Seed Colonized with atoxigenic Aspergillus flavus: characterization and production of a biopesticide for aflatoxin control. Biocontrol Science and Technology. December, 1999. v. 9(4). p. 529-543.

PROCEEDINGS/ABSTRACTS:

Cotty, P.J. Competitive exclusion and aflatoxin control. Presented in the symposium entitled "The Aflatoxin Elimination Program: A Model for Directed Plant Disease Research." National Meeting of the American Phytopathological Society. New Orleans, LA. 2000.

Cotty, P.J. Use of biocontrol by farmer communities to reduce aflatoxin contamination. Presented in the colloquium entitled "Biological Control: A Proven Technology for the New Millenium." General Meeting of the American Society for Microbiology. Los Angeles, CA. 2000.

CRIS Title: Control of Toxic Endophytic Fungi of Corn and Grasses
CRIS: 6612-42000-021
Scientists: Porter JK, Yates ID, Norred WP, Riley RT, Bacon CW, (vacant)
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Summary Project Aims:

Fusarium moniliforme is associated with >1000 plants, with several hundred agriculturally important in the USA. This fungus exists as a symptomless endophyte on several of these, especially corn. During its internal association with the corn plant it produces several mycotoxins. However, this fungus is versatile as it can also colonize dead plant tissue as a saprophyte, and it can colonize kernels in storage where the mycotoxin accumulation initiated in the field can be increased. Thus, this fungus is very unpredictable, and problematic. It is one of a few to have such a versatile nature, and yet be almost universally present in corn and 90% of the isolates are toxic. Because of the endophytic nature, chemical controls of F. moniliforme are difficult and not recommended. We have initiated biocontrols for the endophytic habit and post-harvest mycotoxin accumulation by this fungus. The interactions of the corn plant and the fungus are being examined to determine strategies that could ensure world-wide food safety for corn and its products. Our approach is to use an endophytic isolate of the bacterium biocontrol of the endophytic habit, and to use an isolate of the a species of Trichoderma for biocontrol of the post-harvest aspect. Both the biocontrol bacterium and fungus can be transformed with other desirable genes and more effective biocontrol agents as required. The ultimate solution to this problem of controlling symptomless infections is to utilize and manipulate the genetics of the fungus, and exclude the growth of the fungus in plants, and use molecular technology such as transformations of the biocontrol organisms for increased biocontrol expression in planta (surrogate transformation of the plant). Traditional and molecular approaches will be used to control fungal growth and mycotoxin accumulation in corn and other cereals.

Summary Accomplishments During Entire Project:

The major accomplishment of this CRIS project was identifying and obtaining a patent on a bacterium with enormous biocontrol properties. With this bacterium we can provide the technology to control the endophytic growth and toxin reduction of fungi in corn. This technology is based on the principle of competitive exclusion in which the biocontrol endophytic bacterium, B. subtilis Patent number 5,994,117, replaces and prevents the growth of the fungus inside plants, and provides a significant reduction in fumonisin content in plants. The technology is a patented endophytic bacterium that is unique in internally colonizing plants preferentially, imparting beneficial growth effects as well as controlling the growth of fungi, and reducing toxin accumulation. The long term goal for this technology is to determine the efficacy of this endophytic bacterium and identify others that can be used as control strategies for other corn diseases and mycotoxin controls. We therefore envision a family of bacteria that can be used to protect specific plants, and control other mycotoxins, as well as fungi. Further, this and other endophytic bacteria identified in the research approach also offer the advantages when transformed of being used as vectors of many agricultural biopesticide, agrochemicals and biotransformations of drugs by plants.

This process, surrogate transformation, is being explored as a patented technology by Unit scientists and a CRADA partner. Surrogate transformation should offer an alternative to transgenic plants, but unlike plants the bacterial genome is transformed, simplifying transformed systems considerably.

Summary 2000 Accomplishments:
The endophytic hyphae of the fungus Fusarium moniliforme produces toxins and fungal growth inside plants is very difficult to control. Unit scientists isolated and determined that an endophytic bacterium was effective in preventing the growth of this and other fungi by the mechanism of competitive exclusion. Preliminary tests indicate that it can significantly reduce the mycotoxin accumulation in corn plants. The bacterium also produces growth enhancing characters such as increased root growth and plant growth rate. The finding that this bacterium is unique in being a plant colonizer with biocontrol potential is expected to impact the basic approach of using bacteria as biocontrol strategies for all plants, as well as the use of modified endophytic bacteria to protect and enhance plant growth.

We have identified and developed a new bio-compatible control compound, Plantpro, which has potential for the control of diseases and mycotoxin resulting from systemic fungal infected seed. This compound will have a broad impact on the seed industry, as basil seed have been show to be protected from another species of Fusarium. The iodine-based product kill fungal spores, and inhibits the growth biocontrol nature, and its utility as a commercial technique for protecting seed.

Projected Research Accomplishments During Next 3 Years:
The effects of drought on the accumulation pattern of the fumonisins in plants infected with F. moniliforme will be determined under greenhouse controlled conditions. This study will determine the effects of moderate and severe drought conditions on the accumulation of the fumonisins in plants. This study will also be done in conjunction with the biocontrol bacterium, B. subtilis (=B. mojavensis) as well as its mutants, also determine how this biocontrol bacterium behaves as a protector under moderate and severe drought conditions. The anticipated accomplishment will be establishing the environmental control strategies for the use of the biocontrol bacterium and other endophytic bacteria. Concurrently, The utility of the Trichoderma sp. as a biocontrol in post- and pre-harvest conditions will be examined in an attempt to determine the best parameters that may be utilized for T. viride control of both toxin accumulation and F. moniliforme growth.

Collaboration with our CRADA partner, we expect to have transformed the biocontrol bacterium with desirable genes, and initiated studies to measure the in planta expression of these, as well as the distribution and quantity of the expressed product along the plant axis. Transformed bacteria, obtained from our CRADA partner, will be tested under several conditions of culture. The efficacy of surrogate transformation as a viable tool for protecting plants under greenhouse condition should be established, and the foundation laid for patents for this process.

Technology Transfer:
A patent for the biocontrol bacterium Bacillus subtilis (=B. mojavensis) was granted for the control of diseases caused by fungi. The technology determined by the CRADA should be ready within a 2-4 year period provided information that this technology extends beyond controlling the growth

of the fungus F. moniliforme and other fusaria species in corn. The bacterium can also reduce the mycotoxin produce by fungi. The CRADA has two objectives: to determine the chemical nature of the antifungal substance and surrogate transformation has been initiated for a 2-3 year period. Preliminary data indicate that this substance is a small polypeptide; and to extend this technology to include the use of transformed endophytic bacteria as vectors to transform plants (surrogate transformation) with such genes as the biopesticide genes, and other bioprotective genes.

Another CRADA was established to evaluate a food protectant, a microbial inhibitor, as a growth retardant for F. moniliforme on corn in storage and during its self life. Strains of F. moniliforme are used as the biological assay to determine the use of this microbial inhibitor to control the growth of fungi in general and F. moniliforme in particular. This work is being done under a CRADA with Ajay North America, Inc. The work describing efficacy of the environmentally compatible material in controlling growth of F. moniliforme in the petri dish and in corn seed has been communicated in written reports to the co-operating company, AJAY, Inc.

PUBLICATIONS:

Bacon, C.W., Hinton, D.M. Use of Bacillus subtilis as an endophyte for the control of diseases caused by fungi. Nov 1999. U.S. Patent 5,994,117.

Meredith, F.I. Isolation and characterization of fumonisins. Merrill, A.H., Hannun, Y.A., editors. Academic Press, Inc., New York, NY. Methods in Enzymology. Sphingolipid Metabolism and Cell Signaling Part A. 2000. v. 311. Chapter 37. p. 361-373.

Yates, I.E., Jaworski, A.J. Differential growth of Fusarium moniliforme relative to tissues from 'Silver Queen', a sweet maize. Canadian Journal of Botany. 2000. v.78. p.472-480.

Bacon, C.W., Hinton, D.M. The endophytic host range of Bacillus mojavensis, a new species with biocontrol potential. Phytopathology. June 2000. v.90(6). p.S4.

Glenn, A.E., Bacon, C.W. Detoxification maize antimicrobial compounds by Gibberella fujukuroi. Phytopathology. June 2000. v.90(6). p.S27.

Yates, I., Arnold, J., Hinton, D., Basinger, W. A novel fungicide controls Fusarium moniliforme growth. Phytopathology. June 2000. v.90(6). p.S87.

Bacon, C.W., White, J.F., Jr (ed). Microbial Endophytes. Marcel Dekker, Inc., New York. 2000. 487pp.

Kuldau, G.A., Bacon, C.W. Claviceps and Related Fungi. Hui, Y.H., Smith, R.A., Spoerke, D.G., Jr. editors. Marcel Dekker, Inc., New York. 2000. 503-534.

12.34

CRIS Title: Reduction of Fusarium Mycotoxins as Concerns in Agricultural Commodities
CRIS: 6612-42000-020
Scientists: Voss KA, Porter JK, Bacon CW, Riley RT, Norred WP
Location: Toxicology and Mycotoxin Research, Richard Russell Research Center, Athens, GA
Contact: 706-546-3158 (P); 706-546-3116 (F); cbacon@saa.ars.usda.gov

Summary Project Aims:

This CRIS addresses a number of high priority food safety problems. Several diseases of farm animals are caused by toxic metabolites produced by Fusarium species associated with major crops, including corn and wheat. Fumonisins and other Fusarium toxins could also affect human health, thus the occurrence of adverse amounts of the toxins in food products manufactured from contaminated commodities must be prevented. Mycotoxins can reduce the marketability of crops, especially in export markets where even low-level mycotoxin contamination is used for price leverage. Elimination of the fungi and their mycotoxins from the commodity is the topic of on-going research and is perhaps an ultimate solution. However, there has been little if any success by this method, and the prospects for success are poor. This CRIS attempts to resolve this problem by (1) determining the biochemical and molecular mechanisms of action, and using the knowledge to develop therapeutic agents and bioassay methods for analytical use, diagnostic procedures and decontamination evaluation, (2) determining the effects of processing of Fusarium contaminated corn on mycotoxin content of final products and (3) determining whether Fusarium sp. toxins, alone or in combination, alter the biological activity of other mycotoxins, increase susceptibility to infectious diseases, or produce chronic toxicity. The outcome of this research will be reduction of animal and human health concerns through mycotoxin management that does not depend on elimination of the fungus. Recognition of the high quality of TMRU research in this area ensures USDA representation on panels and committees involved in regulatory activities or in making recommendations to agencies in the U. S. (FDA, EPA), European Union, United Nations (World Health Organization, Food and Agriculture Organization), and other national (Office of Environmental Health Hazard Assessment, Prop. and international organizations (International Life Sciences Institute, International Union for Pure and Applied Chemistry). Another important outcome is that through understanding mechanisms of action, new approaches to control and detection of fungal contamination will be possible.

Summary Accomplishments During Entire Project:

Unit scientists first proposed the molecular mechanism of action of fumonisins in the early 1990's. Studies since then have provided strong evidence that the proposed hypothesis was correct, and that the toxic effects of fumonisins can be explained by their ability to disrupt sphingolipid metabolism. Sphingolipids are important not only as structural components of cells, but also serve as messengers that regulate the activities of cells, including growth, division, differentiation, apoptosis (programmed cell death), and immune response. Research conducted on this CRIS has had a significant impact on the field of sphingolipid biochemistry as well as introducing novel concepts of how fungal metabolites (of which there may be thousands not yet discovered) interact with plants, animals and microorganisms. The discovery has also spurred the search for new antifungal agents to treat the increasing health risks from fungal pathogens.

Unit scientists conducted definitive toxicological studies and were directly involved in the design and successful completion of the National Toxicology Program chronic toxicity and carcinogenicity studies of fumonisin B1 in rats and mice, as well as teratology studies of the mycotoxin in rabbits and rats. Studies showed that fumonisin B1 is carcinogenic in rodents, provided target organ, dose-response and toxicokinetic data, and also showed that fumonisin is not teratogenic. The data is critical for developing risk assessments and regulatory guidelines for fumonisins in food.

Summary 2000 Accomplishments:
For the past ten years the United States has been faced with an economic and health threat that could have resulted in the loss of billions of dollars to the corn industry and consumers due to the widespread presence in corn of fumonisins, toxic chemicals produced by a common mold. ARS's Toxicology and Mycotoxin Research Unit scientists conducted comprehensive studies that revealed the underlying causes and nature of the diseases attributed to fumonisins, and worked with National Toxicology Program collaborators to design, implement and complete long-term studies necessary for risk assessment. The maximum levels in food or feed, which are adequate to protect human and animal health and are now being recommended by the FDA, are a direct result of the research and advisory inputs of the scientists of the Toxicology and Mycotoxin Research Unit. Without the ARS input the projected economic loss to North American dry millers would have been 25 times greater ($270 million vs $11 million) than the loss that would have incurred if the level had been set at the non-scientifically based 1 ppm, as is the case in at least one other country.

Projected Research Accomplishments During Next 3 Years:
Under a CRADA with one of the leading corn chip manufacturers, TMRU will likely complete studies on the fate of fumonisins in corn during food production. Thus far, it appears that processing corn to make masa flour used for tortilla chips significantly reduces the fumonisin content of the flour. This research will demonstrate an added safety factor in the event fumonisin-contaminated corn is used, and will strengthen consumer confidence in corn products. In collaboration with university researchers, it is expected that the promotional and synergistic effects of fumonisins with aflatoxin and nitrosamines will be defined. Since these compounds can co-occur in food and feed, this information is needed to assess the degree of concern warranted by exposure to combinations of toxins. In collaboration with two university researchers, the effects of fumonisins on the immune system will be determined. Fumonisins, through their disruption of sphingolipid biosynthesis, can alter the expression of microbial pathogen and toxin receptors in the gut. Thus the immune response to these agents could be altered by fumonisins, as could the response to vaccines that interact with the same receptors. The research will determine whether or not fumonisins could be involved in microbial diseases or in vaccination failures.

Technology Transfer:
Unit scientists have been requested to share their expertise in numerous national and international food safety forums and to serve as members of organizations that shape the international thinking on the regulation of and human risks associated with mycotoxin contaminated foods. Examples in which scientists presented talks or chaired sessions include scientific symposia, food protection, cereal millers, and seed trade associations.

12.36

PUBLICATIONS:

Lee, K., Barb, C.R., Kraeling, R.R., Riley, R.T., Hartzell, D.L., McGraw, R.A., Azain, M.J., Dean, R.G., Baile, C.A. Expression of beta-galactosidase and pig leptin gene in vitro by recombinant adeno virus. Animal Biotechnology. 1999. v. 10. p.37-48.

Norred, W.P., Bacon, C.W., Riley, R.T., Voss, K.A., Meredith, F.I. Screening of fungal species for fumonisin production and fumonisin- like disruption of sphingolipid biosynthesis. Mycopathologia. 1999 v. 146. p.91-98.

Li, W., Riley, R.T., Voss, K.A., Norred, W.P. Role of proliferation in the toxicity of fumonisin B1: enhanced hepatotoxic response in partially hepatectomized rat. Journal of Toxicology and Environmental Health. 2000. v.60. p. 441-457.

Riley, R.T., Plattner, R.D. Fermentation, partial purification, and use of serine palmitoyltransferase inhibitors from Isaria (=Cordyceps) sinclairii. Merrill, A.H., Hannun, Y.A., editors. Academic Press, Inc., New York, NY. Methods in Enzymology. Sphingolipid Metabolism and Cell Signaling Part A. 1999. v. 311. p. 348-361.

Riley, R.T., Norred, W.P., Voss, K.A., Sharma, R.P., Merrill, Jr., A.H. Fumonisins - occurrence, toxicology and mechanism of action. Kinki University, Kinki, Japan. Bulletin of the Institute of Comparative Agricultural Science. 2000. V.8. p.59-70.

Sharma, R.P., Bhandari, N., Tsunoda, M., Riley, R.T., Voss, K.A. Tolerance to fumonisin toxicity in a mouse strain lacking the P75 tumor necrosis factor receptor. Toxicology. 2000. v.143. p.183-194.

Sharma, R.P., Bhandari, N., Tsunoda, M., Riley, R.T., Voss, K.A., Meredith, F.I. Fumonisin toxicity in a transgenic mouse model lacking the MDR1A/1B P-glycoprotein genes. Environmental Toxicology and Pharmacology. 2000. V.8. p.173-182.

Howard, P.C., Eppley, R.M., Stack, M.E., Warbritton, A., Voss, K.A., Lorentzen, R.J., Kovach, R., Bucci, T.J. Carcinogenicity of fumonisin B1 in a two-year bioassay with Fischer 344 rats and B6C3F1 mice. Mycotoxins. 1999. v.S99. p. 45-54.

Voss, K.A., Porter, J.K., Bacon, C.W., Meredith, F.I., Norred, W.P. Fusaric acid and modification of the subchronic toxicity to rats of fumonisin in F. moniliforme culture material. 1999. Food and Chemical Toxicology. V. 37. p. 853-861.

PROCEEDINGS/ABSTRACTS:

Voss, K.A., Porter, J.K., Bacon, C.W., Meredith, F.I., Norred, W.P. Fusaric acid did not modify the hepatic or renal toxicity of fumonisin-producing Fusarium moniliforme. Society of Toxicology, New Orleans, LA, March, 1999. Toxicological Sciences. 1999. v.48(1S). p. 54.

Enongene, E.N., Sharma, R.P., Voss, K.A., Riley, R.T. Comparison of sphingolipid changes in two strains of mice dosed with fumonisin B1 (FB1) by two different routes. Society of Toxicology, Philadelphia, PA. March 2000. Toxicological Sciences. 2000. v.54. p.142.

Riley, R.T., Showker, J.L., Voss, K.A., Enongene, E.N., Meredith, F.I., Sharma, R.P. Fumonisin B1-induced liver toxicity, liver free sphinganine and lipid peroxidation compared in six different strains of male mice. Society of Toxicology, Philadelphia, PA. March 2000. Toxicological Sciences. 2000. v.54. p.307.

Dresden-Osborne, C., Noble, G.P., Enongene, E.N., Riley, R.T., Bacon, C.W., Voss, K.A. The effect of fumonisin producing Fusarium moniliforme on selected immune responses of mice to Trypanosoma cruzi. Society of Toxicology, Philadelphia, PA. March 2000. Toxicological Sciences. 2000. v.54. p.142.

Riley, R.T., Merrill, Jr., A.H. Inhibitors of sphingolipid biosynthesis as research tools for understanding the mechanism of action of fumonisins. Society of Toxicology, Philadelphia, PA. March 2000. Toxicological Sciences. 2000. v.54. p.243.

Bhandari, N., Enongene, E.N., Riley, R.T., Meredith, F.I., Sharma, R.P. Time related tumor necrosis factor - alpha (TNF alpha) expression and signaling due to fumonisin B1 in vivo. Society of Toxicology, Philadelphia, PA. March 2000. Toxicological Sciences. v.54. p.387.

Sharma, R.P., Bhandari, N., Riley, R.T., Voss, K.A., Meredith, F.I. Fumonisin Toxicity in tumor necrosis factor 2 (TNFR2) knockout mice. Society of Toxicology, Philadelphia, PA. March 2000. Toxicological Sciences. v.54. p.387.

Sharma, R.P., Riley, R.T., Voss, K.A. Cytokine involvement in fumonisin-induced cellular toxicity. p. 109. Presentation at the International Union of Pure and Applied Chemistry, Guajuna, Sao Paulo, Brazil, June 2000.

Norred, W. P., Riley, R.T. Mechanism of action of mycotoxins. p. 102. Presentation at the International Union of Pure and Applied Chemistry, Guajuna, Sao Paulo, Brazil, June 2000.

12.38

CRIS Title: Agronomic, Environmental, and Resistant Germplasm Effects on Aflatoxins and other Mycotoxins
CRIS: 6402-42000-001
Scientists: Abbas HK, Meredith WR, Bruns HA
Location: Crop Genetics Production Research, Stoneville, MS
Contact: 662-686-5313 (P);662-686-5218 (F); habbas@ars.usda.gov

Summary Project Aims:
Corn production in the Mississippi Delta has returned after a hiatus of nearly 30 years. Demand for corn by both poultry and catfish producers has created a market for locally grown grain. Corn has potential as an important rotational crop in the Mid South with cotton and soybeans. Aflatoxins produced by Aspergillus flavus and A. parasiticus, are known hepatic toxins carcinogens and can occur on corn grains under certain environmental conditions. Contamination of corn with aflatoxin has been severe under drought conditions and has caused serious economic losses in the Mid South. Fumonisins, produced by Fusarium species, have more recently been discovered to cause leukocencephalomalacia in horses, pulmonary edema in swine, and is also tumor promotor. These compounds can be present in large amounts and are becoming a regulatory and food safety issue. The purpose of this project is to significantly reduce mycotoxin contaminations in corn in the Mid South with particular emphasis on aflatoxins. Research is being done on plant populations, irrigation, harvest dates and planting dates, using corn hybrids currently available to Mid South growers. Hybrids adapted for production in the northern most parts of the Corn Belt are also being examined for their production potential in the Delta. The effects these practices have upon mycotoxin presence are being evaluated. Studies are also being conducted to determine possible hybrid differences in susceptibility to fungal infection.

Summary Accomplishments During Entire Project:
Again, this project is new this year and specific results were summarized in the previous question. We plan to develop methods of reducing mycotoxin contamination in the corn crop in order to make this a viable alternative crop in the Southern U.S. Testing varieties of corn for the presence of fumonisins and aflatoxins will lead to recommendation of preferred varities for resistance to toxin formation. Studies of cultural practices such as till and no-till, planting dates and irrigated vs. non-irrigated plots will enable us to make recommendations to farmers in order to minimize mycotoxin contamination. The soil survey has led to a collection of toxigenic and non-toxigenic A. flavus isolates. These will be used to conduct studies to determine if non-toxigenic isolates will compete with toxigenic isolates and thus reduce toxin contamination. These interventions have the potential to save up to $30 million in current losses and to expand the use of corn in the South.

Summary 2000 Accomplishments:
This is the first year of existence for this CRIS. Research into the effects of plant populations, harvest dates, planting dates, corn-cotton rotations and early maturing corn production systems have been initiated. Preliminary results indicate that some early maturing corn hybrids may be profitably produced in the Mid South and that further research is warranted to determine this. A preliminary study on the presences of mycotoxins in corn grown in Mississippi in 1998 and 1999 will be presented at the American Phytopathological Society meeting in August, 2000. A cooperative agreement with the University of Minnesota has allowed confirmation of the presence of

fumonisins, specifically FB1, FB2, FB3, FB4, and FC4. Soil samples from corn fields (conventional and no-till) in the Mississippi Delta were collected and propagules of Fusarium and Aspergillus were isolated. These isolates were screened for toxin production. All F. moniliforme isolates produced fumonisins, but only 60% of A. flavus produced aflatoxins. These data will be used in further studies to determine if non-toxigenic isolates compete with toxigenic isolates to reduce mycotoxin contamination.

Projected Research Accomplishments During Next 3 Years:
We are developing corn production systems that will be profitable and sustainable for the Mid South. These systems take into account the fact that available equipment, fertility needs, planting dates, genetic resources, pest and other production concerns for the Mid South are different from what is found and utilized by corn growers in other parts of the United States. Research concentrated on determining optimum plant populations, planting depths, planting dates, corn/cotton rotations, effects of delayed harvest, refining early maturing production systems, refining fertility recommendations for corn that meet the crop's nutritional needs and environmental constraints, minimizing irrigation inputs and reducing the incidence of mycotoxin contamination.

Field experiments are being conducted evaluating adapted and arly season corn hybrids for resistance to aflatoxin and Fumonisin contamination. Developing corn ears were injected with A. flavus and F. moniliforme to be evaluated for synergistic, antagonistic or no competitive effect by the fungi. Other experiments involve testing 4 hybrids (two Bt and two non-Bt) for resistance to aflatoxin contamination by different inoculation techniques (spraying, needle injection and toothpick inoculation). This will allow us to determine possible hybrid differences in A. flavus susceptibility and the most useful technique for mass inoculations in future experiments. Soil samples from corn fields in the Mississippi Delta using various tillage systems, cover crops and irrigated vs. non-irrigated production systems were taken and analyzed for propagates of toxigenic and non-toxigenic isolates of A. flavus. These data will be correlated to determine if cultural practices influence mycotoxin contamination in the mature grain.

Research initiated in 2000 will be continued and barring any unforeseen difficulties, sufficient data on plant populations, depth of planting and date of harvest should be collected to make sound recommendations to growers. Data from early maturing systems should be sufficient to plan expansion of the study and take a more aggressive approach to developing a viable production strategy for these hybrids. Sufficient data on some studies will be available for manuscript preparation. Research on date of planting and corn/cotton rotations will be continued. Experiments on aflatoxin and fumonisin - resistant corn hybrids will be continued and fine-tuned according to data collected in the previous year. More varieties will be tested from breeding programs from Mississippi State and Baton Rouge when they are developed. After the toxigenic and non-toxigenic isolates are catalogued, these isolates will be tested to see if it is possible to overcome toxigenic isolates with non-toxigenic. We will also test cropping systems to see if fungal contamination can be manipulated by different cultural practice. Research on nutrient uptake will likely be expanded to help refine fertility recommendations for corn production. Such research will not only benefit producers by maximizing net income but previous research on mycotoxin production have shown that proper fertilization of corn greatly reduces the incidence of aflatoxin. Studies to develop viable production systems using early maturing corn hybrids will likely be initiated. A study designed to

determine the potential yield of corn in the Mid South is under consideration and will likely be initiated. Such a study will determine what the maximum yield of corn is and give researchers a base on which to work towards in selecting yield goals.

Technology Transfer:
Because this is a new project, no technology transfer has occurred yet. In 2001 and 2002, the outcome of the experiments should be useful to farmers and seed companies.

PUBLICATIONS:

Abbas, H.K., Cartwright, R.D., Xie, W., Mirocha, C.J., Richard, J.L., Dvorak, T.J., Sciumbato, G.L., Shier, W.T. Mycotoxin Production by Fusarium proliferatum isolates from rice with Fusarium sheath rot disease. Mycopathologia 1999. v. 147, p 97-104

Abbas, H. K., Cartwright, R.D., Windham, G.L., Xie, W., Shier, W.T., Mirocha, C.J. The presence of mycotoxins and fungi in rice and corn in the southern United States. Bull. Inst. Compr. Agric. Sci. Kinki Uni. 2000. v 8, p 23-38

Abbas, H. K., Smemda, R.J., Gerwick, C., Shier, W.T. Fumonisin B1 from the fungus Fusarium moniliforme causes contact toxicity in plants. Evidence from studies with biosynthetically labelled toxin. Journal of Natural Toxins 2000. v 9, p 85-100

Shier, W. T., Resch, P., Badria, F., Abbas, H.K. Biological Consequences of Fumonisins. Bull. Inst. Compr. Agr. Sci. Kinki Univ. 2000. v.8. p.71-78.

Shier, W. T., Tiefel, P.A., Abbas, H.K. Current research on mycotoxins: Fumonisins. ACS Symposium Series, In: Natural and Selected Synthetic Toxins--Biological Implications, Eds. A. T. Tu and W. Gaffield. 2000. Chapter 5. p 54-66

Shier, W. T., Abbas, H.K. Current issues in research on fumonisins, mycotoxins which may cause nephropathy. J. Toxicol. Toxin Rev. 2000. v.18. p.323-335.

PROCEEDINGS/ABSTRACTS:

Abbas, H.K., Duke, M.V., Shier, W.T., Riley, R.T., Merrill, A.H., and Duke, S.O. 2000. The structure-activity relationships of fungal of toxins that disrupt sphingolipid biosynthesis in plant and animal systems. Third International Weed Science Congress-IWSC, Brazil, June 6-11, 2000.

CRIS Title: The Use of Aflatoxigenic Strains of *Aspergillus flavus* to Prevent Aflatoxin Contamination
CRIS: 5344-42000-012
Scientists: Henneberry T
Location: Western Cotton Research Laboratory, Phoenix, Arizona
Contact: 602-437-0121 (P);602-437-1274 (F); thenneberry@wcrl.ars.usda.gov

Summary Project Aims:
Aflatoxins are carcinogenic toxins produced by members of the Aspergillus flavus group of fungi. There are currently no methods that growers can apply in order to reliably and economically prevent aflatoxin content of crops from exceeding mandated levels. It has recently become apparent that the aflatoxin producing potential of fungi resident in agricultural areas might be reduced through competitive exclusion by atoxigenic strains. However, the utility of atoxigenic strains in commercial agriculture needs to be determined and methods are needed to optimally utilize atoxigenic strains both regionally and locally. A process for production of commercially useful quantities of high-quality atoxigenic strain inoculum is needed in order to adequately test and develop field aspects of atoxigenic strain technology. Development of a production process that is also economical is necessary to create a potential for this technology to become commercially useful.

Summary Accomplishments During Entire Project:
This project was initiated during 2000. A pilot-scale process for production of atoxigenic strain inoculum was developed and is in the process of being tested. Personnel employed by the Arizona Cotton Research and Protection Council were trained in aseptic techniques and to implement the process. Initial assessment of the utility of atoxigenic strains in diverse habitats and under varying agronomic practices is underway.

Summary 2000 Accomplishments:
Commercial scale use of atoxigenic strain technology as a biological ontrol of aflatoxin contamination, large quantities of atoxigenic strain material are needed. A pilot scale process capable of producing 1,400 lb. of material per day was developed by ARS in collaboration with the Arizona Cotton Research and Protection Council and is under evaluation at pilot facility under construction in Arizona. This process is the second phase of the scale-up and retains the initial criterion of being able to be run by a relatively low technology grower organization. The second phase pilot-process was used to produce over 100,000 lb. of atoxigenic strain inoculum used to treat over 10,000 acres of cotton included in trials directed at developing protocols for area-wide aflatoxin management programs based on atoxigenic strain technology. Atoxigenic strains may become the first practical technology for limiting aflatoxin contamination on the farm.

Projected Research Accomplishments During Next 3 Years:
We expect to design and assemble the third phase pilot-scale process and to use the resulting information to develop a full-scale process. We hope to use the resulting data to develop a prototype full-scale process. Protocols usable by grower organizations and acceptable to EPA will be develop

for quality control and each step of fungal spore handling. Initial commercial field area-wide evaluations will be completed and impediments to successful utilization of the technology in Arizona and elsewhere will be defined as research questions requiring attention.

Technology Transfer:
Technologies associated with utilizing atoxigenic strains of A. flavus to prevent aflatoxin contamination are in the process of being transferred to the Arizona cotton producing community. The development and assessment of area-wide implementation of atoxigenic strains is being performed with the cotton industry throughout Arizona. Processes for manufacturing the biocontrol agent will be transferred to Arizona grower organizations and to organizations from other states (Texas is a particular target) for use with locally adapted strains. The process details will also be made available as a public sector technology for adaptation to the production of other biological control agents.

PUBLICATIONS:

Bock, C.H., Cotty, P.J. Wheat Seed Colonized with atoxigenic Aspergillus flavus: characterization and production of a biopesticide for aflatoxin control. Biocontrol Science and Technology. Dec 1999. v. 9(4):529-543

PROCEEDINGS/ABSTRACTS:

Cotty, P. J. "Competitive exclusion and aflatoxin control," presented in the symposium entitled "The Aflatoxin Elimination Program: A Model for Directed Plant Disease Research" at the National Meeting of the American Phytopathological Society, New Orleans, LA, 2000

Cotty, P. J. "Use of biocontrol by farmer communities to reduce aflatoxin contamination," presented in the colloquium entitled "Biological Control: A Proven Technology for the New Millenium" at the Annual Meeting American Society Microbiology, Los Angeles, CA, 2000

CRIS Title: Reduction of Aflatoxin in Tree Nuts Through Control of Insect Pests Using Natural Products
CRIS: 5325-42000-031
Scientists: Campbell BC, Light DM, Roitman JN, Buttery RG, Wong R.
Location: Plant Protection Research Unit, WRRC, Albany, CA
Contact: 510-559-5846 (P); 510-559-5777 (F)

Summary Project Aims:
Aflatoxin contamination of tree nuts (almonds, pistachios and walnuts) is a major food safety and foreign trade issue. Tree nuts are valued at approximately $2 billion/ yr. Aflatoxin is reported to be one of the most potent natural carcinogens known and is a sensitive food safety issue. 50-70% of tree nuts are exported and action levels for aflatoxin in foods by major importing nations threaten the tree nut industry. Safe methods must be developed to reduce insect damage to tree nuts, which leads to aflatoxin contamination.

Summary Accomplishments During Entire Project:
A number of potentially effective plant odors have been isolated and identified for use in controlling tree nut pests. Methods to implement these compounds to reduce use of pesticides in tree nut orchards are underway. Compounds were identified that inhibit the conversion of aflatoxin to a carcinogen.

Summary 2000 Accomplishments:
Insects damage tree nuts leading to aflatoxin contamination. Use of pesticides to control insect pests of tree nuts is costly, fiscally and environmentally. ARS researchers designed and field tested implementation strategies using newly discovered plant volatiles for control of codling moth, a major pest of walnuts and pome fruits. Tests were done in walnut, apple and pear orchards in collaboration with colleagues at USDA-YARL, U. California, Berkeley and an industrial CRADA partner. A new plant odor was found to be as attractive to codling moth as the sex pheromone but is attractive to female moths which lay eggs that hatch into larvae which do the feeding damage. This odor can be combined with a pesticide to lure only codling moth and thus eliminate the need for widespread application of pesticides which can lead to contamination and negative impacct to non-target organisms.

Aflatoxin is converted into a carcinogen in human liver. We wanted to find safe, natural chemicals that occur in foods that could inhibit the formation of this carcinogen. Of more than 100 natural compounds tested, five very promising compounds were discovered that inhibit carcinogen formation in the test tube. Some of these discovered compounds are known to be found in human liver after ingestion. The potential exists that these anti-cancer products could be added to foods that might run the risk of being contaminated with aflatoxin, especially a problem in developing countries. Research will benefit both small farms and farm workers, to control insects at a reduced expense and reduce risks of exposure to toxic pesticides.

New Natural Plant Odor Proves To Be Significant New Tool In The Control of Codling Moth. Codling moths are one of the most destructive agricultural pests to walnut, apple and pear. A natural plant odor was discovered that is a potent lure on codling moth. This odor is now being used to

attract moths to baits which are impregnated with pesticides. This method of control will only kill the codling moths, does not kill other organisms, including beneficial ones, and reduces risk of people being exposed to pesticides.

Projected Research Accomplishments During Next 3 Years:
Continue search for plant odors identified in fruit and nuts for other tree nut pests. Test attracticides against codling moth. Isolate natural compounds which inhibit formation of carcinogenic or toxic compounds from aflatoxin. Test compounds for effectiveness in liver. Search for compounds capable of degrading aflatoxin.

Technology Transfer:
Technology transfer is underway through a CRADA agreement with an industrial partner. Together, we are developing monitoring, trapping and attracticide systems using plant odors against codling moth and in other tree nut pests. These will be used as a method to disrupt the insects from being able to mate.

PUBLICATIONS:

Wollenweber, E., Dorr, M., Roitman, J.N. Epicuticular flavonoids of some Scrophulariaceae. Z. Naturforsch. 2000. v. 55c. p. 5-9.

Buttery, R.G., Light, D.M., Nam, Y., Merrill, G.B., Roitman, J.N. Volatile components of green walnut husks. Journal of Agriculture Food Chemistry. 2000. v. 48 (7). p. 2858-2861.

Molyneux, R. J., Mahoney, N., Campbell, B. C. Anti-aflatoxigenic constituents of Pistacia and Juglans species. In: Natural and Synthetic Toxins. Biological Implications. Tu, A. T. and W. Gaffield, editors, ACS Symposium Series. 2000. v. 745, p. 43-53.

PROCEEDINGS/ABSTRACTS:

Campbell, B.C., Lee, S.-E., Light, D.M., Mahoney, N., Merrill, G., Molyneux, R., Roitman, R.N. Tree nut - aflatoxin interactions: Natural products affecting insect pests, growth of Aspergillus, aflatoxigenesis and aflatoxin biotransformation. Proceedings of the 11th Aflatoxin Elimination Workshop, Atlanta, GA. 2000. p. 39-41.

Mahoney, N.E., Molyneux, R.J., Campbell, B.C., McGranahan, G.H., Gradziel, T.M. Comparison of aflatoxin production on defatted tree nuts: High anti-aflatoxigenic activity in walnut. Proceedings of the 11th Aflatoxin Elimination Workshop, Atlanta, GA. 2000. p. 55.

Campbell, B.C., Heraty, J., Rasplus, J.-Y., Chan, K., Steffen-Campbell, J.D., Babcock, C. Molecular systematics of the Chalcidoidea using 28S-D2 rDNA. Austin, A. D. and Dowton, M., editors. CSIRO, Melbourne. 2000. The Hymenoptera: Evolution, Biodiversity and Biological Control. p. 57-71.

CRIS Title: Inhibition of Tree Nut Contamination by Aflatoxins and Related Mycotoxins using Natural Products
CRIS: 5325-42000-032
Scientists: Campbell BC, Molyneux RJ, Bayman P, Hua SST
Location: Plant Protection Research Unit, WRRC, Albany, CA
Contact: 510-559-5846 (P); 510-559-5777 (F)

Summary Project Aims:
Contamination of tree nuts by aflatoxins produced on infection by certain fungi is a serious problem because of potential threat to human health. The current domestic guideline set by the FDA is 20 ppb total aflatoxins but the European Union (EU) has instituted a standard of 2 ppb aflatoxin B1 and 4 ppb total aflatoxins. The aggregate value of almonds, pistachios and walnuts is currently over $2 billion, and 60-70% of the crop is exported. This regulation thus constitutes a major threat to US exports of these crops. Research is being conducted to identify natural constituents of nut crops that provide resistance to infection by aflatoxin producing fungi and reduce the ability of the fungus to produce aflatoxin. We are also identifying yeasts that are biocompetitive towards these fungi to be used as biological control agents.

Summary Accomplishments During Entire Project:
Kernels of the walnut variety, Tulare, have been shown to possess the ability to completely suppress aflatoxin formation in laboratory experiments. These results should permit the identification of specific resistance factors and establish the pattern of heritability since the parentage of this variety is known. Studies have shown that rehydration to facilitate cracking of closed-shell pistachios results in exceptionally high aflatoxin levels and that this process should be abandoned. A series of natural chemicals called naphthoquinones, which occur in walnuts, have been shown to be inhibitors of aflatoxin production; this provides a useful tool to elucidate mechanisms of resistance. Saprophytic yeasts have been discovered that are biocompetitive to aflatoxin producing fungi. Successful field trials of these yeasts will provide a means to displace aflatoxin producing fungi from the tree nut environment.

Summary 2000 Accomplishments:
Aflatoxins, the cancer-causing products of mold growth, can severely limit the marketability of tree nuts in both domestic and export markets and there is a need to identify natural resistance factors which will prevent their formation. Laboratory experiments showed that walnuts were much more resistant to aflatoxin formation than almonds and pistachios and that the Tulare variety of walnut completely suppressed aflatoxin production, with the resistance factor being confined almost entirely to the thin, paper-like skin (pellicle) surrounding the kernel of the nut. This is the first example of a commercial variety of any crop plant known to be contaminated by aflatoxins that shows complete resistance. Since the pellicle is inherited from the maternal parent, it should be possible to develop new crosses with predictable, heritable natural resistance to aflatoxigenesis.

Research Accomplishments During Next 3 Years:
Experiments will be conducted to identify the natural resistance factors to aflatoxin contamination present in Tulare variety walnuts and a pattern of heritability will be established. This information will be extended to almonds and pistachios. A comprehensive database of distribution of mycotoxin producing fungi in tree nuts will be developed. The genetic, physiological and morphological variation of toxin producing fungi in tree nut orchards will be elucidated and their relationship to toxin production defined. The ecological relationship between biocompetitive yeast strains and Aspergillus flavus strains present in tree nut orchards will be studied to determine the factors essential for effective biocontrol.

Technology Transfer:
Pistachio processors have been informed that rehydration in order to facilitate cracking of closed-shell pistachios stimulates post-harvest aflatoxin formation and should not be employed. The avoidance of this technique can be instituted immediately by processors.

PUBLICATIONS:

Molyneux, R. J., Mahoney, N., Campbell, B. C. Anti-aflatoxigenic constituents of Pistacia and Juglans species. In: Natural and Synthetic Toxins. Biological Implications. Tu, A. T. and W. Gaffield, editors, ACS Symposium Series. 2000. v. 745, p. 43-53.

Hua, S.-S. T., Grosjean, O.-K., Baker, J. L. Inhibition of aflatoxin biosynthesis by phenolic compounds. Letters in Applied Microbiology 1999. v.29, p. 289-291.

Watson, A.A., Davies, D.R., Asano, N., Winchester, B., Kato, A., Molyneux, R.J., Stegelmeier, B. L., Nash, R. J. Calystegine. Alkaloids in the potato and other food plants. In: Natural and Synthetic Toxins. Biological Implications. Tu, A. T. and W. Gaffield, editors, ACS Symposium Series. 2000. v. 745, p. 129-139.

Panter, K. E., Gardner, D. R., James, L. F., Stegelmeier, B. L., Molyneux, R. J. Natural toxins from plants affecting reproductive function in livestock. In: Natural and Synthetic Toxins. Biological Implications. Tu, A. T. and W. Gaffield, editors, ACS Symposium Series. 2000. v. 745, p. 154-172.

Asano, N., Nash, R. J., Molyneux, R. J., Fleet, G. W. J. Sugar mimic glycosidase inhibitors: Natural occurrence, biological activity and prospects for therapeutic application. Tetrahedron: Asymmetry. 2000. v. 11, 1645-1680.

de Balogh, K. K. I. M., Dimande, A. P., van der Lugt, J. J., Molyneux, R. J., Naudé, T. W., Welmans, W. G. A lysosomal storage disease induced by Ipomoea carnea in goats in Mozambique. Journal of Veterinary Diagnostic Investigations. 1999. v. 11, p. 266-273.

Stegelmeier, B. L., James, L. F., Panter, K. E., Gardner, D. R., Pfister, J. A., Ralphs, M. H., Molyneux, R. J. Dose response of sheep poisoned with locoweed (Oxytropis sericea). Journal of Veterinary Diagnostic Investigations. 1999. v. 11, p. 448-456.

PROCEEDINGS/ABSTRACTS:

Campbell, B.C., Lee, S.-E., Light, D.M., Mahoney, N., Merrill, G., Molyneux, R., Roitman, R.N. Tree nut - aflatoxin interactions: Natural products affecting insect pests, growth of Aspergillus, aflatoxigenesis and aflatoxin biotransformation. Proceedings of the 11th Aflatoxin Elimination Workshop, Atlanta, GA. 2000. p. 39-41.

Hua, S.-S. T., Du, W., Payne, G. A., Flores-Espiritu, M., Baker, J.L. Repression of GUS reporter constructs of the aflatoxin biosynthetic pathway genes by phenolic compounds. Proceedings of the 11th Aflatoxin Elimination Workshop, Atlanta, GA. 2000. p. 47.

Mahoney, N. E., Molyneux, R. J., Bayman, P. Effect of almond processing on viability of A. flavus spores. Proceedings of the 11th Aflatoxin Elimination Workshop, Atlanta, GA. 2000. p. 53.

Mahoney, N. E., Molyneux, R. J., Campbell, B. C., McGranahan, G. H., Gradziel, T. M. Comparison of aflatoxin production on defatted tree nuts: High anti-aflatoxigenic activity in walnut. Proceedings of the 11th Aflatoxin Elimination Workshop, Atlanta, GA. 2000. p. 55.

Hua, S.-S. T. Biological control research to reduce aflatoxin in almonds and pistachios. Proceedings of the 11th Aflatoxin Elimination Workshop, Atlanta, GA. 2000. p. 81.

Hua, S.-S. T., McAlpin, C. E., Baker, J. L., Platis, C. E. Characterization of the Aspergillus flavus population from a California tree nut orchard. Proceedings of the 11th Aflatoxin Elimination Workshop, Atlanta, GA. 2000. p. 92.

Hua, S.-S. T., Baker, J. L., Flores-Espiritu, M. Control of Aspergillus flavus in contaminated almonds by saprophytic yeasts. Proceedings of the 11th Aflatoxin Elimination Workshop. 2000. p. 93.

Hua, S.-S. T. Biocontrol approach to reduce Aspergillus flavus population in tree nut orchards. Proceedings of the California Conference on Biological Control, M. S. Hoddle, editor. July, 2000. p. 147-150.

12.48

CRIS Title: Removal of Aflatoxin Contamination from Human Foods in Real Time by Imaging Techniques
CRIS: 5325-42000-030
Scientists: Schatzki TF, Keagy PM, Pearson TC
Location: Cereal Product Utilization Research Unit, WRRC, Albany, CA
Contact: 510-559-5672 (P); 510-559-5777 (F)

Summary Project Aims:
Tree and ground nuts, like many food products, can become infected with molds, either before harvest or during storage. In nuts, some of these molds produce a mycotoxin, aflatoxin, which is among the most potent liver carcinogens known. When present, aflatoxin is contained in a very few nuts, but these infected nuts carry individually a very high level of toxin. Sampling lots for such contamination is a serious problem as it is statistically quite likely that good lots are rejected and bad ones accepted, simply due to sampling errors. We have developed methods to characterize these aflatoxin distributions. Using the distributions we can estimate the likelihood of sampling errors. However, very large samples (hundreds of pounds) may be required with standard methods. Another, more desirable, solution to the problem is to sort the nuts before final shipment, removing the contaminated ones. We are developing automatic sorting methods that remove contaminated nuts from the process stream.

Summary Accomplishments During Entire Project:
Over the eight years of the current and preceding projects, we have established methods to ascertain how much and where aflatoxin occurs in the process streams of almonds and pistachios. We have developed sorting equipment which can reduce the aflatoxin level in pistachios below measurable levels. The pistachio industry has followed our suggestions or improved processing and is now installing these sorters. Increased sales in Europe alone amounted to $30m last year. We have found methods of detecting insects or insect damage within nuts which, at least in the case of almonds, should reduce aflatoxin levels in that commodity as well. A rapid laboratory method of sorting peanuts for high aflatoxin content has been developed and been applied to develop a training set of infected peanuts as a step in developing a peanut sorter.

Summary 2000 Accomplishments:
Analysis of pistachios currently requires a very large nut sample to reduce sampling errors. We have developed a calibrated 3-step sampling plan for pistachios that reduces the amount of nuts and testing effort required to characterize a lot for aflatoxin. Use of this sampling plan with an average sample of 23 lbs. results in the same precision in aflatoxin determination as the usual 1-step test using 110 lbs. of nuts. Adoption of this plan will save the industry $1.0 M/year in sample and test costs. Mechanical sorter to remove high aflatoxin peanuts from the processing stream is currently not available. A set of peanuts, that is known to contain high aflatoxin levels, has been used to train a projected peanut sorting machine. The selection method involves dipping peanuts in a solution and testing the solution for high aflatoxin levels by use of mass spectroscopy, while the peanuts themselves are subsequently dried in vacuum. Successful development of a high speed sorter will allow elimination of aflatoxin from peanuts, a major food source in much of the world.

Projected Research Accomplishments During Next 3 Years:
A quantitative relation between insect infestation and flatoxin levels in walnuts will be completed. Work to identify possible Properties for peanut sorting should commence. Work on the peanut sorting system will commence. Work on the peanut sorting system should be completed.

Technology Transfer:
Pistachio sorting for aflatoxin based on visible imaging, has been licensed and installations in pistachio processing plants are expected to increase. We are told that major pistachio processors are operating their plants guided by the distribution and sorting papers we have published in the last five years. The sequential sampling test method for pistachios has been taught to the California Pistachio Commission, which is recommending it to the industry as a standard method. Further development of machines for quality nut sorting is continuing under in another CRIS. A paper on buyer's and seller's risk in pistachios, published in 1999, is expected to strongly influence the testing of pistachios in the European Union. In all these areas, market and cost constraints operate.

PUBLICATIONS:

None

PROCEEDINGS/ABSTRACTS:

Schatzki, T.F., Pearson, T.C., Haddon, W.F. Post harvest selection and sorting for aflatoxin in tree and ground nuts. Aflatoxin Elimination Workshop, Atlanta, GA, October, 1999.

Schatzki, T.F. Testing for aflatoxin concentration in pistachios by sequential sampling. Presented to the California Pistachio Commission, March, 2000.

CRIS Title: Poisoning of Livestock by Various Larkspur (*Delphinium*) Species
CRIS: 5428-32630-008
Scientists: Pfister JA, Gardner DR, Ralphs MH, Stegelmeier BL, Panter KE, James LF, Lee S
Location: Poisonous Plant Research Laboratory, NPA, Logan, UT
Contact: 435-752-2941 (P); 435-753-5681 (F); lfjpprl@cc.usu.edu

Summary Project Aims:
Larkspur (*Delphinium* spp.) poisoning causes serious economic loss to livestock producers whose cattle graze on many western ranges. The objectives of the multidisciplinary research are: 1. isolate and identify individual alkaloids from *Delphinium* species; test these alkaloids for toxicity; confirm structure-activity relationships for new alkaloids; 2. develop analytical and diagnostic methods for larkspur alkaloids, including development of immunogenic alkaloid-protein conjugates and antibody-based detection methods and potential vaccine development; 3. investigate mechanisms of intoxication, including toxin metabolism and clearance, and treatment of intoxicated animals; and 4. determine plant, animal, or environmental factors influencing larkspur consumption and continue development of management strategies to reduce losses for all classes of larkspur.

Summary Accomplishments During Entire Project:
Developed the Fourier transform infrared spectroscopic method of analysis of toxic and total alkaloid levels in tall larkspur, thus allowing for rapid analysis of thousands of samples. Elucidated the comparative toxicity of 24 alkaloids from larkspurs and structure activity relationships. Understanding toxicity has important implications for developing rational treatments to reduce losses. Developed grazing strategies to reduce losses to tall larkspurs. Continued implementation of early- and late-season grazing strategies has resulted in reduced cattle losses for ranchers whose cattle graze on tall larkspur-infested pastures. Determined that cattle eat tall larkspur in a cyclic "toxification-detoxification" pattern after ingesting a daily threshold of 14-18 mg toxic alkaloid/kg b.w. This research helps to understand patterns of consumption and how much larkspur is necessary to poison cattle. Determined that when cattle eat larkspur, adverse post-ingestive consequences regulate consumption, not plant flavor (i.e., taste and smell). Increases in the toxic alkaloid concentration deter consumption by sheep, but have little impact on short-term consumption by cattle. This research provides fundamental information about when and why cattle eat larkspurs. Diet selection studies showed that cattle grazing high mountain ranges often respond to temperature changes during and immediately after storms by rapidly consuming large amounts of tall larkspur. Such gluttonous consumption probably accounts for many cattle deaths in areas of dense larkspur populations. Determined that mineral supplementation does not affect the amount of larkspur consumed by grazing cattle, indicating that producers can save money by providing mineral supplements as necessary for nutritional needs, rather than using minerals to alter larkspur consumption. Developed aversive conditioning as a potentially viable tool for ranchers. This practice could potentially greatly reduce the risk of cattle losses for those livestock producers that implement aversive conditioning. Developed herbicidal control recommendations for tall larkspurs. Ranchers with dense populations of larkspur can greatly reduce losses and enhance economic returns through the use of herbicides. Verified that sheep grazing before cattle can reduce larkspur availability and acceptability to cattle grazing afterward.

Collaborative work with Colorado State University investigated the mechanism of action of MLA, nudicauline, 12-deacetylnudicauline, barbanine, and deltaline. It was determined that the alkaloids block nicotinic acetylcholine (nAch) receptors postsynaptically. The potency of deltaline is much lower, indicating that deltaline does not bind as tightly as do the other alkaloids at nAch receptors.

Summary 2000 Accomplishments:
Current tall larkspur taxonomic classifications are confusing and impede the flow of clear management recommendations based on individual species. This research at PPRL, Logan, Utah developed a systematic approach to the taxonomic classification of tall larkspurs based on molecular genetics, plant morphology and alkaloid profiles. This systematic approach showed that there are 3 distinct tall larkspur species, and management recommendations can now be tailored to each individual species. These taxonomic classification and related management recommendations will be used by other scientists, land management agencies (e.g., USFS) livestock producers, and veterinarians to better diagnose and solve tall larkspur poisoning problems.

Determining the alkaloid profiles of larkspur alkaloids is essential to determine potential toxicity. This research completed the study of mass spectrometry analysis of toxic larkspur alkaloids. This methodology is being used to identify new alkaloids in tall and low larkspurs and was the basis for a chemotaxonomy investigation of three tall larkspur species. This chemical analysis will benefit other scientists, land management personnel that deal with larkspurs, and livestock producers that need assistance to determine toxicity of larkspurs.

Three competitive inhibition enzyme-linked immunosorbent assays (CI-ELISA) for toxic larkspur alkaloids were developed. One assay is class specific toward the *N*-(methylsuccinimido) anthranoyllycoctonine (MSAL) alkaloids, while two assays are specific for individual alkaloids. The assay with the lowest limit of detection had an I_{50} of 191 pg with a limit of detection of 30.5 pg for methyllycaconitine. Spike and recovery studies using bovine blood and brain tissue ranged from 52 - 89 %, suggesting that with additional development these techniques are likely to be excellent tools to diagnose poisoned animals and identify highly toxic plants.

Vaccine development continues with the testing of three conjugates developed from larkspur alkaloids. Trials are currently underway in mice and preliminary results look promising. Toxicokinetic work on larkspur alkaloids also is continuing. The primary toxic alkaloid, MLA,. is apparently secreted unchanged in the urine, and $T_{1/2}$ in most tissues is about 6 hrs. Defoliation of tall larkspur reduced its vigor and toxic alkaloid pools for two years. Further research will be conducted to determine if simulated grazing will reduce toxicity.

Projected Research Accomplishments During Next 3 Years:
Develop and refine equations to predict toxicity of given larkspur populations. Continue support of alkaloid analyses for rangeland or toxicity studies. Continue immunization and toxicokinetic studies with mice. Continue development of grazing management strategies for low and plains larkspurs. Complete the publications on the chemotaxonomy of tall larkspurs. Complete low larkspur toxic alkaloid survey. Determine toxicokinetics (absorption and elimination profiles) of larkspur alkaloids

in a sheep model. Begin study of larkspur-induced bloat in cattle. Begin to study relationship between precipitation and abundance of low larkspur populations. Determine effects of simulated grazing on toxic alkaloids in tall larkspur.

Continue ELISA and immunization studies. Continue development of grazing management strategies for low/plains larkspurs. Continue investigation of toxicokinetics (absorption and elimination profiles) of larkspur alkaloids in a sheep model, and analyze blood, urine and feces for alkaloids. Continue study of larkspur-induced bloat in cattle. Continue to study relationship between precipitation and abundance of low larkspur populations.

Complete study of larkspur-induced bloat in cattle. Continue ELISA and immunization studies. Continue to study relationship between precipitation and abundance of low larkspur populations. Complete toxicokinetic study in sheep.

Technology Transfer:
Toxic larkspur alkaloid analyses done on a number of plant samples for ranchers and extension personnel. This is a useful tool for ranchers to help determine if they can begin grazing with low risk, or continue to graze, a particular pasture or allotment. The management concepts referred to as "primary toxic window" and "early season grazing have been developed and transmitted to numerous ranchers and USFS personnel. These concepts are based on plant chemistry and cattle diet selection patterns, and are being used to reduce losses on summer-grazed Forest Service allotments where tall larkspur is problematic.

Organized symposium on "Do most livestock losses to poisonous plants result from 'poor' range management" at Society for Range Management Meeting Feb., 2000 (Eight, 30-minute presentations also submitted for publication in Journal of Range Management). Organized and presented "Tall larkspur management" workshop for livestock producers and agency personnel in Steamboat Springs, CO. March, 2000. Workshop sponsored by Colorado State University Extension Service.

PUBLICATIONS:

Dobelis, P., Madl, J.E., Pfister, J.A., Manners, G.D., Walrond, J.P. Effects of *Delphinium* alkaloids on neuromuscular transmission. Pharmacology Experimental Therapeutics. 1999. 291: 538-546.

Gardner, D.R., Panter, K.E., Pfister, J.A., Knight, A.P. Analysis of toxic norditerpenoid alkaloids in *Delphinium* species by electrospray, atmospheric pressure chemical ionization, and sequential tandem mass spectrometry. Agriculture Food Chemistry. 1999. 47: 5049-5058.

Gardner, D.R., Pfister, J.A. Late season toxic alkaloid concentrations in tall larkspur (*Delphinium* spp.). Journal of Range Management. 2000. 53:329-334.

Gardner, D.R., Manners, G.D., Panter, K.E., Lee S.T. Pfister, J.A. Three new toxicnorditerpenoid alkaloids from the low larkspur *Delphinium nuttallianum*. Natural Products. 2000. 63:1127-1130.

Ralphs, M.H., Gardner, D.R., and Pfister, J.A. A functional explanation for patterns of norditerpenoid alkaloid levels in tall larkspur (*Delphinium barbeyi*). J. Chemical Ecology. 2000. 26: 1595-1607.

CRIS Title: *Astragalus* and *Oxytropis* Poisoning in Livestock
CRIS: 5428-32000-008
Scientists: James L, Ralphs M, Stegelmeier B, Panter K, Pfister J, Gardner D, Lee S.
Location: Poisonous Plant Research Laboratory, Logan, UT
Contact: 435-752-2941 (P); 435/753-5681 (F); lfjpprl@cc.usu.edu

Summary Project Aims
Toxic species of the *Astragalus* and *Oxytropis* genera poison livestock and adversely affect the harvesting of forage by livestock. These species cause significant poisoning problems in the western U.S. and Canada, South America, and China. Objectives of the research are: 1. Develop techniques to diagnose locoweed poisoning and better prognose the fate of poisoned animals. Describe the lesions of locoweed poisoning in deer and elk and compare locoweed-induced lesions with those of chronic wasting disease.2. Determine dose and time locoweed can be grazed before reproductive functions become compromised. 3. Refine analytical methods to detect and quantify swainsonine in plant material, and determine the source of variability in the presence of swainsonine in locoweed species. 4. Synthesize swainsonine or swainsonine analogs and their protein conjugates, determine if these conjugates are immunogenic, and develop immunologic diagnostic techniques and vaccines. 5.Determine the conditions under which locoweeds are grazed and poisoning occurs. Develop grazing management strategies to avoid critical periods and management tools to reduce the risk of poisoning. 6. Describe locoweed population cycles; determine the influence of climate and weather on population outbreaks and die-offs. Determine if competition from cool-season grasses will suppress locoweeds. 7. Determine the toxic effects of various forms of selenium (sodium selenate, organic selenium and plant selenium) on reproduction in cattle. Further differentiate the various type of Se intoxication i.e. acute alkali disease and blind staggers.

Summary Accomplishments During Entire Project:
Identified the indolizidine alkaloid swainsonine as the toxin in locoweed. International symposium "Swainsonine and Related Glycosidin" held at Logan, Utah. The clinical symptoms of poisoning were described. Extended information of locoweed on reproduction, i.e. abortions, birth defects, cessation of oogenesis, spermatogenesis, weak and underweight lambs, etc. Demonstrated that cattle grazing white locoweed at high elevations experienced increased incidence of congestive heart failure. Showed that swainsonine is excreted in milk and poisoning of nursing young results. Fetal lambs and calves develop the same histological lesions as the dam grazing locoweed. Documented the immunologic effects of chronic locoweed poisoning in cattle. Poisoning initially stimulates lymphocyte proliferation and immunoglobulin secretion, chronic poisoning results in decreased immune function. Poisoning appears to be a result of tissue swainsonine concentration. Tissues that accumulate high swainsonine develop lesions at lower locoweed doses. Once a tissue develops lesions, histologic changes do not become more severe with increasing locoweed doses. Toxicity is a result of α-mannosidase and mannosidase II inhibition. Once all available enzyme is inhibited, lesion severity does not increase with increasing swainsonine consumption. Developed enzymatic assays for swainsonine and determined swainsonine toxicokinetics. Swainsonine is rapidly cleared from serum and skeletal muscle (T½~20 hours), hepatic and renal tissues (T½= 60 hours), and a recommended withdrawal time of 28 days (10 T½'s) for poisoned animals. Serum α-mannosidase

activity and swainsonine concentrations can be used as indicators of locoweed poisoning in livestock. As serum swainsonine and α-mannosidase activities return to normal after animals discontinue eating locoweed, both have limited clinical usefulness. Pulmonary intravascular macrophages and myocardial interstitial cells are sensitive to swainsonine. Lesions in these cells contribute to increased pulmonary vascular hypertension and congestive heart failure.

Determined that locoweeds (*Astragalus lentiginosus* and *Oxytropis sericea*) cause reproductive dysfunction in livestock; in rams reduced libido, caused testicular degeneration, sperm abnormalities, and altered sexual behavior. Recent research demonstrated that a resazurin dye assay to measure ram sperm metabolic activity after exposure to locoweed is indicative of reproductive potential in rams exposed to locoweeds. Recovery of spermatogenesis may occur, but permanent neurological deficits may preclude normal reproductive function. While heifers poisoned on locoweed recovered their reproductive function, the effects on the estrous cycle and ovarian function were significant and occurred relatively soon after locoweed ingestion began. Determined that locoweed caused a reduction in fetal heart action resulting in fetal death and abortion. This maybe a contributing factor in hydrops (water belly) in cattle and subsequent abortion.

Examined the effects of locoweed consumption during gestation and lactation on ewe and lamb behavior. Lambs were compromised at birth, as could not nurse properly. Previously intoxicated ewes and impaired neonates did not bond. Behavioral deficits in lambs did not persist beyond about 4 weeks. Lambs' abilities to acquire and retain a taste aversion were not altered by *in utero* locoweed intoxication. Determined that maternal consumption of locoweed did not influence young lambs to begin eating locoweed, but consumption by young lambs predisposed them to consumption of locoweed later in life. Examined the behavioral impacts of "on-off" feeding of locoweed followed by recovery, and determined that sheep never fully recovered behaviorally from the initial toxic insult even though pathological lesions resolved.

Identified the conditions of locoweed grazing and subsequent poisoning on mountain summer range, desert winter range, short-grass prairies, and pinyon/juniper range, and management strategies have been developed. Procedures were developed to aversely condition cattle to eating locoweed. This information has been transferred to ranchers. Cattle wintering on wheat pasture and mineral nutrition had no effect on locoweed consumption or intoxication. Prevention lies in restricting access to locoweeds when they are relatively more palatable than associated forages. Locoweed intoxication causes weight loss in stocker cattle, gains do not resume for several weeks after cattle stop grazing locoweed. Management recommendations were developed to prevent poisoning:

Described poisoning from nitro-toxins in *Astragalus* species. Described the chemical/taxonomic relationships between the sections of *Astragalus* containing 3-nitropropanol or 3-nitropropanoic acid form of the toxin. A qualitative analysis of nitro-toxins in *Astragalus* plants developed based on Fourier transform infrared spectroscopy, to screen plants for these toxins. Demonstrated that seleniferous forage does not produce the blind staggers condition as described in the literature, but do cause acute and chronic poisoning. Compared selenium poisoning in cattle fed sodium selenate, *A. bisulcatus* and sodium sulphate.

Summary 2000 Accomplishments:
Completed the development of an analytical method for the analysis of swainsonine in plant material. It will allow us to monitor toxin concentration in plant and animal tissues. We described the immunologic effects of chronic locoweed poisoning in cattle. Cattle developed normocytic normochromic anemia, white blood cell vacuolation, increases in certain serum enzymes, and decreases in serum iron, transferrin and thyroid hormones. Serum proteins showed marked dose-dependant glycosylation changes. Flow cytometry studies showed irregularity decreases in CD4 and CD8 positive lymphocyte during locoweed dosing. Initially, low dose locoweed treatments had a mild stimulative effect on lymphocyte blastogenesis. No differences were detected in the ability to react to injected antigens or in cell mediated response, but locoweed treated animals had significantly increased IgA secretion. These findings suggest chronic locoweed poisoning causes significant immunologic changes.Horses were averted to white locoweed. Lithium chloride gastric lavage at 190 mg/kg b.w. averted horses to fresh locoweed.

Swainsonine is not cytotoxic to oocytes, or early embryos. *In vitro* techniques, showed swainsonine did not alter bovine oocyte maturation, fertilization rate or embryo growth and development. Therefore, locoweeds' adverse effects on conception and early growth and development of embryos is due to effects on the maternal reproductive system secondarily affecting oocyte maturation and embryo development.

Projected Research Accomplishments During Next 3 Years:
Develop diagnostic techniques such as specific glycosalation of serum proteins as indicators of locoweed poisoning. Grazing studies on *Astragalus lentiginosus* (specklepod locoweed) with cattle and horses investigate supplemental feeding of protein and energy on locoweed selection. Determine if swainsonine is produced by an endophyte on *Oxytropis lambertii*. Conclude the comparative study of locoweed poisoning and CWD in deer. Using archival material, compare the effects of locoweed on horses, cows, sheep, goat, deer and elk. Determine the impact of locoweed feeding on conception, early embryonic growth and development in sheep. Determine hormonal levels in follicular fluid from cystic ovaries in locoweed heifers. Develop ELISA technology to detect swainsonine in plant and animal tissue. Test additional swainsonine-protein conjugates for use as vaccines.

Technology Transfer:
Swainsonine analysis made available to livestock producers, veterinarians, diagnosticians, research scientists, and governmental agencies through collaboration with Utah State Diagnostic Laboratory. A summary of the aversion research to prevent livestock from eating poisonous plants was presented to the British Nutrition Society. A summary was published in Rangelands, outlining procedures to implement aversions on ranches. Presented a workshop on "Locoweed poisoning in livestock" to producers, agency and extension personnel at Steamboat Springs, CO.

13.8

PUBLICATIONS:

Panter, K.E., James, L.F., Stegelmeier, B.L., Ralphs, M.H., Pfister, J.A. Locoweeds: Effects on reproduction in livestock. Journal of Natural Toxins. 1999. 8:53-62.

Ralphs, M.H., and James, L.F. Locoweed grazing. 1999. Journal Natural Toxins. 1999. 8:47-51.

Ralphs, M.H., Graham, D., Duff, G., Stegelmeier, B.L., and James, L.F. Impact of locoweed poisoning on grazing steer weight gains. Journal Range Management 2000. 53:86-90.

Ralphs, M.H. and Provenza, F.D. Conditioned food aversions: principles and practices, with special reference to social facilitation. Proc. Nutrition. Society 1999. 58:813-820.

Stegelmeier, B.L., James, L.F., Panter, K.E., Gardner, D.R., Pfister, J.A.., Ralphs, M.H., Molyneux, R.J. The response of sheep poisoned with different doses of locoweed (Oxytropis sericea). Journal Veterinary Diagnostic Investigation. 1999. 11:448-456.

Stegelmeier, B.L., James, L.F., Panter, K.E., Ralphs, M.H., Gardner, D.R., Molyneux, R.J. and Pfister, J.A. The pathogenesis and toxicokinetics of locoweed (Astragalus and Oxytropis spp.) poisoning in livestock. Journal of Natural Toxins. 1999. 8:35-46.

Stegelmeier, B.L., Edgar, J.A., Colegate, S.M., Gardner, D.R., Schoch, T.K., Coulombe, R.A. and Molyneux, R.J. Pyrrolizidine alkaloid plants, metabolism and toxicity. Journal of Natural Toxins. 1999. 8:95-116.

CRIS Title: Livestock Poisoning by Pyrrolizidine Alkaloids and Other Hepatotoxic and Teratogenic Plants
CRIS: 5428-32000-009
Scientists: Gardner DR, Stegelmeier BL, James LF, Panter KE, Pfister JA, Lee S.
Location: Poisonous Plant Research Laboratory, Logan, UT
Contact: 435-752-2941 (P); 435-753-5681 (F); lfjpprl@cc.usu.edu

Summary Project Aims:
Plants containing hepatotoxic pyrrolizidine alkaloids (PA' s) frequently invade pastures and fields and they may contaminate feeds, food and herbal preparations. The resulting livestock, wildlife and human poisonings impair human and animal health and impede trade. Research is being conducted to describe the clinical and pathological alterations of PA intoxications in animals; develop diagnostic procedures; develop knowledge of toxin metabolism and the effects of low dose exposures; develop screening and toxin identification technology to ensure quality food animal products; and determine conditions under which animals are poisoned and develop techniques to avoid or minimize the effects of these poisonous plants. Lupines and other teratogenic plants continue to cause livestock losses in the Western U.S. Catastrophic losses have been reported on individual ranches in broad regional areas of the west. Research on other teratogenic and neurotoxic plants is being conducted to identify toxins, describe plant-induced lesions, and delineate the mechanisms of action of these toxic species.

Summary Accomplishments During Entire Project:
The toxicity of various PA-containing plants including *Senecio*, *Crotalaria*, *Cynoglossum* and *Amsinkia* has been studied and documented. Techniques have been developed to isolate and identify PA in plant and animal tissues. Methods and management schemes such as herbicide control and using less susceptible species such as goats and sheep to control PA-containing plants have been developed.

The specific periods of pregnancy when cattle are susceptible to the teratogenic effects of lupines have been defined. Skeletal malformations and occasional cleft palate occur when lupines are ingested during 40 to 70 days gestation. This teratogenic period was extended to day 100 using epidemiology information gained from field cases in Oregon. The specific period of gestation when cleft palate may be induced was defined in goats to be 35 to 41 days and in cattle 40 to 50 days. Research on the mechanism of action of the skeletal malformations and cleft palate has been accomplished using a goat model. This model and the information gained from cleft palate induction has been transferred to Brown University in Providence, Rhode Island, to study the formation of cleft palate in humans and to develop surgical methods to repair these cleft palates during mid pregnancy when scarring is minimal or absent.

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Summary 2000 Accomplishments:
Several PA-protein conjugates were synthesized, injected into sheep and found to be immunogenic. The antisera produced were tested for specificity to PA and PA metabolites. ELISA's and other techniques were developed to monitor PA concentrations in blood and plant material. These developments are promising as such class-specific assays could be of great use in screening foods, feeds, and herbal products for PA contamination.

Several studies were completed to better determine the fetal and neonatal effects of low dose PA exposure. As pigs are especially sensitive to PA intoxication (humans have similar toxicity), low dose exposure of pyrrolizidine alkaloids and effects on neonates are being explored using a pig model. An initial pilot feeding program was completed testing the use of PA plant material and purified alkaloids. Chemical methods for detection of PA in liver samples from intoxicated animals were improved and used to analyze samples from the pig pilot study. No correlation was found between dose and detectable liver metabolites. Toxicity was found to be most severe for neonatal pigs that are 3 weeks old. Oral toxicity is similar for freebase and N-oxide PA's. In collaboration with the Utah State Animal Disease Diagnostic Laboratory, numerous samples from livestock producers, veterinarians, diagnosticians and other government agencies were analyzed for PA metabolites (pyrroles). Pyrroles were detected in liver from free ranging bison, suggesting these animals are at risk to develop PA-induced disease.

Work studying plant-induced birth defects continued with further development of our plant-induced models of craniofacial, palatal, and skeletal defect models. Two additional lupines (*L. sulphureus* and *L. arbustus*) were shown to be teratogenic in cattle. In collaboration with surgeons from Brown University, pregnant animals carrying fetuses with plant toxin-induced palatal defects were used to develop new in utero surgical repair techniques. Initial reviews suggest these techniques are highly effective in repairing these defects with no scar formation. As such defects are common in human fetuses, these techniques will be of great worth for human surgeons and patients.

Projected Research Accomplishments During Next 3 Years:
During 2001 manuscripts describing the ELISA's and effects of low dose PA exposure will be prepared. Additional studies will be done to determine the toxicity of PA metabolite (pyrrole)/tissue adducts. Pyrroles are PA metabolites that are likely to be found in tissues of poisoned animals. The toxicity of rayless goldenrod (*Haplopappus heterophyllus*) will be determined in horses. We will complete characterization of the goat model with researchers at Brown University and Rhode Island Hospital to study the mechanism of cleft palate induction and development of surgical techniques to repair the cleft palates in utero during gestation. In 2002 pyrrole protein adducts will be synthesized and injected into test animals. If immunogenic, the resulting antibodies will be tested for specificity and avidity. ELISA and immunofluorescent techniques using these antibodies will be developed. The effects of rayless goldenrod will be documented in pregnant and lactating mares. Craniofacial measurements will be completed using the goat model to compare surgical repair of cleft palate during mid gestation with neonatal goats repaired at 6 weeks of age. Using *in vitro* fertilization and embryo culture techniques, we will determine cytotoxic effects of various teratogenic alkaloids on cleavage rates and early embryo development. In 2003, rats will be

immunized with various PA and pyrrole protein conjugates. Dosing with purified PA will test the susceptibility of these immunized rats. Comparison of histopathology of the palatal shelves and mandibular and neck muscles will be done at different stages of gestation in fetal goats after induction of cleft palate with plant toxins.

Technology Transfer:
Information has been transferred to other scientists and researchers through publications in peer-reviewed journals and to scientists, land managers, extension agents and ranchers through popular publications, seminars, work shops, e-mail and telephone conversations. Techniques of tissue analysis for PA metabolites (pyrroles) have been published. The assay is available for livestock producers, veterinarians, and diagnosticians through the Utah State Animal Disease Diagnostic Laboratory. Spectrometric and chromatographic PA detection techniques have been published and used by toxicologists, scientists and researchers.

Techniques to surgically repair cleft palate in the fetus in utero have been developed in a goat model and published. This technique has the potential to be used in human cases of fetal surgery where cleft palate compromises the survival of the fetus. A goat model has been characterized to study the induction and formation of cleft palate and this model and information therefrom has been transferred to researchers at Brown University and Rhode Island Hospital. This model is being used to develop surgical techniques to repair cleft palates in utero during mid-gestation. These techniques will be used in repair of cleft palates in human fetuses during mid-gestation when scarring is minimal or nonexistent, thus preventing or reducing the need for post-natal surgery.

PUBLICATIONS:

Stegelmeier, B.L., Edgar, J.A., Colegate, S.M., Gardner, D.R., Schoch, T.K. Coulombe, R. A. and Molyneux, R. J. Pyrrolizidine alkaloid plants, metabolism and toxicity. Journal Natural Toxins. 1999. 8:95-116.

Schoch, R.K, Gardner, D.R., Stegelmeier, B.L. GC/MS/MS detection of pyrrolic metabolites in animals poisoned with the pyrrolizidine alkaloid riddelliine. Journal Natural Toxins. 2000. 9:97-206.

Panter, K.E., Weinzweig, J., Gardner, D.R., Stegelmeier, B.L. and James, L.F. Comparison of Cleft Palate Induction by *Nicotiana glauca* in Goats and Sheep. Teratology. 2000. 61:203-210.

PROCEEDINGS/ABSTRACTS:

Gay, C.C., Motteram, E., Parish, S., Pritchett, L., Cleasby, J.,Lovely, D. and Panter, K.E. Difference in lupine ingestion by cattle as a risk factor for Lupine-induced crooked calf disorder and possible management by reducing exposure to lupine early in pregnancy. Grazing Conference, 1999, Moscow, Idaho.

13.12

CRIS Title: *Pinus* and *Gutierrezia* Species: Toxicoses and Abortion in Livestock
CRIS: 5428-31320-002
Scientists: Panter KE, James LF, Gardner DR, Pfister JA, Ralphs MA, Stegelmeier BL, Lee S.
Location: Poisonous Plant Research Laboratory, NPA, Logan, UT
Contact: 435-752-2941 (P); 435-753-5681 (F); lfjpprl@cc.usu.edu

Summary Project Aims:
Grazing of ponderosa pine needles causes abortion/premature parturition, retained placentas and endometritis in pregnant cattle resulting in substantial economic losses to cattle producers in the western U.S. and Canada. Lodgepole pine and Monterey Cypress have also been implicated in abortion in cattle. Similar losses occur in the southwestern U.S. when cattle, sheep and goats graze broom snakeweed. Resolutions to these problems are being addressed from a multi-disciplinary approach requiring research on five main objectives: 1. Isolate and characterize the abortifacient and toxic components in pine needles and broom snakeweed. 2. Determine the absorption, metabolism and clearance of abortifacient compounds and describe their physiological activity. 3. Describe the pathophysiology, endocrine, immunologic and morphologic changes associated with abortion and define how pine needles/broom snakeweed cause the abortions. 4. Evaluate potential vaccines, antitoxins and toxin scavengers and determine what treatments can be applied to reduce losses from premature offspring and retained placentas. 5. Determine animal, environmental, and management factors influencing consumption and develop strategies to reduce losses. Most cattle operations are considered small farms (under $250,000 annual gross receipts) and this research will provide information and technology to reduce losses and subsequently improve economic stability to these producers and the rural areas where they live.

Summary Accomplishments During Entire Project:
The most significant accomplishment over the life of the project has been the isolation and identification of the abortifacient toxin in pine needles as the labdane resin acid named isocupressic acid (ICA). After extensive extractions and fractionation experiments two ICA derivatives, succinyl and acetyl ICA were also found to be abortifacient, however, it was determined that succinyl and acetyl ICA are converted to ICA in the rumen of cattle. Isocupressic acid itself is metabolized in the rumen and after absorption. The rumen and serum metabolites of ICA have been identified. The four major metabolites are dihydroagathic acid, agathic acid, imbricatoloic acid, and tetrahydroagathic acid. Maximum serum metabolite concentrations occur between 24 and 48 hours after consumption of pine needles. Compounds structurally similar to ICA have been identified in broom snakeweed but the exact abortifacient toxin has not been identified yet. Broom snakeweed also contains many other compounds that are overtly toxic to cattle.

Isocupressic acid (ICA) was also detected in *Cupressus macrocarpa* (monterey cypress), *Pinus contorta* (lodgepole pine), and *Juniperus communis* (common juniper). In subsequent feeding trials lodgepole pine and common juniper were demonstrated to be abortifacient. Twenty-three tree and shrub species from throughout the western and southern states were analyzed for ICA. Significant levels (>0.5% dry weight) were detected in *Pinus ponderosa*, *P. jefferyi*, *P. contorta* (lodgepole

pine), *Juniperus scopulorum* (Rocky Mountain juniper) and *J. communis* (common juniper). Identification and measurement of the toxic chemicals in pine needles can provide useful information for the range scientist and ranchers in formulating management practices to prevent losses.

Ponderosa and lodgepole pine needles, common juniper and *Cupressus macrocarpa* (an abortifacient tree from New Zealand containing ICA) were also analyzed for the presence of vasoactive lipids including myristate and laurate esters of 1,14-tetradecanediol and 1,12-dodecanediol. These vasoactive lipids have been proposed as possible abortifacient components in pine needles. Concentration of these vasoactive lipids were 0.028%, 0.023%, 0.001% and ND (none detected) for ponderosa, lodgepole, common juniper and *C. macrocarpa,* respectively, confirming that these lipids are not required to induce pine needle-induced abortion in cattle.

Grazing and pen studies have examined conditioned food aversions as a potential tool to keep cattle from consuming pine needles. Cattle were successfully averted to green needles initially, but the aversion extinguished as cattle began consuming dry needles and then green needles. In nutrition trials, low energy status initially influenced cows to eat more needles but as cows gained experience with needles this treatment effect diminished; supplemental protein did not result in increased pine needle consumption.

Summary 2000 Accomplishments:
Research to provide immune-based analytical assays and vaccines for the abortifacient toxin in pine needles (ICA) will improve diagnoses and treatment for these toxicoses. At the Poisonous Plant Research Lab in Logan, UT isocupressic acid-protein conjugates were prepared using ovalbumin (OVA, MW~43,000) for investigation of potential immune-based assays (ELISA) and vaccines to better diagnose and reduce or prevent abortion in cattle from ponderosa pine needles. Isocupressic acid (ICA) is a small molecule and not naturally immunogenic therefore, ICA was conjugated to ovalbumin (OVA, MW~43,000) to elicit an immune response, and is a significant first step towards development of assays (ELISA) or vaccines. Outcome of this phase of research would be improved diagnostic technology and potential treatment or prevention of pine needle/broom snakeweed abortions in cattle.

Seasonal changes in isocupressic acid levels were monitored in ponderosa pine at five locations and no significant differences were observed in needle isocupressic acid levels. There were significant differences among individual trees and locations but differences were not correlated to tree age.

Seven different treatments were evaluated, including hormonal therapy, calcium channel blockers, prostaglandin inhibitors, metabolic enhancers, etc., to investigate potential biochemical mechanisms of action of pine needle-induced abortions. Developed therapies and treatment for the post-abortion sequelae such as retained placentas and endometritis in cows and enhanced premature calf survival. Research will continue to test effects of cow nutrient status and food offering on intake of pine needles.

The effects of high and low energy and protein on consumption of pine needles by pregnant cows was studied. All cows ate substantial amounts of pine needles, but cows consuming high energy diets ate more pine needles than cows on low energy rations; protein levels in diets had no major effect on pine needle consumption.

Feeding corn silage has been reported to reduce amounts of pine needles eaten by pregnant cattle. A study was done to determine if corn silage altered pine needle consumption in pregnant cattle. Silage given ad libitum to cattle had no major effect on pine needle consumption, but near the end of the study some cattle did begin to show a decrease in consumption of needles due to silage.

Using monensin has been touted as a means to reduce pine needle consumption by cattle. A study was done to determine if 200 mg/hd/day of monensin supplementation would alter pine needle intake by pregnant cows. Monensin had no effect on pine needle consumption in treated cows compared to untreated controls.

Projected Research Accomplishments During Next 3 Years:
we expect to complete the write-up and report results of seasonal and location effects on ICA levels in ponderosa pine needles. Appropriate ICA or ICA metabolites for conjugation and development of immunologic active compounds for vaccine trials will be selected. The immunologic activity of ICA conjugates will be established and tested for cross reactivity to the ICA metabolites. The small-scale synthesis of isocupressic acid metabolites will be started. Nutritional studies of supplemental feeding regimes designed to reduce consumption of pine needles by grazing cattle will be conducted. Broom snakeweed plants from different populations will be collected for subsequent diterpene acid profiling and preliminary trials to compare aversion to dry and green needles will be conducted.

We will start the profiling of diterpene acid content of different broom snakeweed populations and explore scale up synthesis of ICA metabolites. We will continue investigation of immunologic activity of ICA and continue evaluation of vaccine in cows. The nutritional status of cows and subsequent propensity to graze pine needles and broom snakeweed will continue to be defined. We will continue work on averting cattle to PN using both green and dry needles and start a grazing study on relationship between forage availability/quality and pine needle consumption. We will plan the scale-up synthesis of ICA metabolites for animal trials and continue investigating the toxic and abortifacient activity of broom snakeweed. Grazing studies on the relationships between forage availability/quality and pine needle consumption will be continued.

Technology Transfer:
Information has been transferred to other scientists through publications in peer-reviewed journals and to scientists, land managers, extension agents and ranchers through popular publications, seminars, work shops, e-mail and telephone conversations.

PUBLICATIONS:

Stegelmeier, B.L., James, L.F., Gardner, D.R., Panter, K.E. and Pfister J.A. 1999. Pine Needle Abortion In Cattle. Cattle Producer's Library-Cooperative Extension System.

Panter, K.E., Gardner, D.R., James, L.F., Stegelmeier, B.L. and Molyneux, R.J. *In*: Natural And Selected Synthetic Toxins: Biological Implications (A.T. Tu and W. Gaffield, eds). American Chemical Society, Washington, DC, 2000. p. 154-172.

13.16

CRIS Title: Prevent the Occurrence of Toxins in Water to Protect Food and the Environment.
CRIS: 5442-42000-003
Scientists: Larsen GL, Garber EAE, Hakk H, Shelver WL, Shappell NW.
Location: Animal Metabolism-Agricultural Chemicals Research Unit, Red River Valley Agricultural Research Center, Fargo, ND
Contact: 701-239-1231 (P); 701-239-1430 (F); larseng@fargo.ars.usda.gov

Summary Project Aims:
Identify and quantify biologically active compounds (BACs, e.g. toxins, mycotoxins, phytoestrogens, estrogens, or other endocrine disrupting materials) in fresh water sources that effect food safety, agricultural activity and the food chain. Areas of focus are the identification of hormonally active agents, drugs, and other naturally occurring compounds from a variety of known or unknown sources. These compounds may be associated with amphibian declines and deformities or may result from animal feeding operations or municipal sewage treatment plants. We have obtained water samples from lakes in which deformed frogs have been found in Minnesota and Vermont. Additional samples will be obtained from manure composting and stockpiling sites and sites near municipal sewage treatment plants this fall. Lake samples will be studied to determine possible agent(s) responsible for the frog malformations. Water samples from manure handling areas and surrounding areas will be studied for estrogen levels to determine estrogen load on the environment. Levels of pharmaceuticals in the environment will also be determined.

Summary Accomplishments During Entire Project:
The presence of malformed frogs in MN lakes may be an indication that hormonally active agents such as endocrine disruptors are present in fresh water sources, which in turn may have a negative impact on agriculture and the food chain. Water samples collected from 16 lakes and analyzed using the FETAX bioassay with additional screening for the effects of mineral supplementation, population density, and for the presence of volatile hormonally active agents correlated with the occurrence of malformed frogs and frog population declines. An evanescent field fluorometric biosensor (Judith Erb, ThreeFold Biosensors, Ann Arbor, MI) has shown a strong correlation with the FETAX results. The impact of this research has lead to a receptor based purification methodology which used in conjunction with GC/MS and NMR should allow the isolation and identification of the causative agent(s) and provides valuable information regarding the cause of malformed frogs and frog population declines. 17-β-Estradiol and testosterone are potent estrogenic and androgenic substances present in large quantities in animal waste, which have the potential to become environmental endocrine disruptors. Analyses using a commercial enzyme immunoassay kit of these hormones in samples related to manure handling practices showed a) high hormone levels are present in water adjacent to manure compost piles, b) some degradation of these hormones in manure occurs when allowed to stand at -20°C, c) the hormones are readily water soluble at native levels which means they can be transported by rainfall/runoff, d) commercial manure products contain these hormones, occasionally in relatively large amounts, and e) the hormones can be transported through a soil column into a groundwater system. These results show that the environment contains a

background level of potent estrogens and androgens. The impact of these hormone levels in soil and ground water needs to be evaluated in terms of potential toxicity and detrimental developmental outcomes in the environment.

Summary 2000 Accomplishments:
The occurrence of malformed frogs in MN lakes may be an indication that hormonally active agents, such as endocrine disruptors, are present in fresh water sources, which in turn may have a negative impact on agriculture and the food chain. Through a collaboration with Joe Magner of the Minnesota Pollution Control Agency (MPCA, St. Paul, MN), water samples were collected from 16 lakes and analyzed using the FETAX (Frog Embryo Teratogenic *Xenopus*) bioassay with additional screening for the effects of mineral supplementation, population density, and for the presence of volatile hormonally active agents. The results obtained from the FETAX assay correlated with the occurrence of malformed frogs and frog population declines and indicated that there are BACs in water from effected lakes, which cause developmentally delayed embryos in the FETAX assay. These results were presented at several meetings, and three manuscripts are under preparation. The ubiquitous presence of endocrine disruptors, such as estrogenic compounds, has become a potentially serious threat to the food chain and public health.

Screening for estrogenic compounds was conducted in collaboration with Judith Erb (ThreeFold Biosensors, Ann Arbor, MI) using a novel evanescent field fluorometric biosensor that employs the human Estrogen Receptor-a along with ELISA analyses for 17-ß-estradiol and testosterone. The biosensor results displayed a strong correlation with the FETAX results and the occurrence of deformed frogs and declines in frog populations; a receptor based affinity purification methodology, in conjunction with GC/MS and NMR, is currently being developed to isolate and identify the causative agent(s). The arylhydrocarbon receptor (AhR) and the retinoic acid receptor (RAR) provide sensitive methodologies for the detection of several groups of potentially serious endocrine disruptors, such as coplanar PCBs and retinoic acids. As part of a collaboration with Randy Allen (Hybrizyme Inc., NC) and Elwood Linney (Duke Univ.), a competitive AhR binding assay and a developmental, RAR transgenic zebra assay were employed to screen water samples from lakes in MN. Preliminary results are inconsistent with the presence of compounds capable of interacting with either the AhR or the RAR. Amphibian malformation and decline have been reported in the Midwest but causative factors have not been fully elucidated. Therefore, we conducted a field/lab study to determine the rate of mortality and malformation of the northern leopard frog (an affected species) at two sites in MN, one known to contain malformed frogs and the other "unaffected." This was done in collaboration with MPCA who assisted in conducting field surveys of wild populations of frogs. We found extensive mortality at both sites, while no mortality was found in lab-raised tadpoles/frogs or those raised at the ND site of origin. Of enclosure frogs successfully achieving metamorphosis, only one was observed with a malformation, while "wild" tadpoles and frogs of species that overwintered as tadpoles (green and mink frogs) exhibited malformations. A fungus, saprolegnia, has been implicated in the mortality at one site, while the transmittable biological agent at the other site is unknown. These results will help MPCA and other wildlife agencies develop strategic plans to monitor frog populations and malformations.

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The native hormones, 17-β-estradiol and testosterone, are among the most potent estrogenic and androgenic substances known and are eliminated in large amounts in animal waste to perhaps become environmental endocrine disruptors. We analyzed samples related to manure handling practices for the levels of these native hormones by utilizing a commercial enzyme immunoassay kit. Our results show that a) high hormone levels are present in water adjacent to manure compost piles, b) some degradation of these hormones in manure occurs when allowed to stand at -20°C, c) the hormones are readily water soluble at native levels, which means they can be transported by rainfall/runoff, d) commercial manure products contain these hormones, occasionally in relatively large amounts, and e) the hormones can be transported through a soil column into a groundwater system. These results show that the environment contains a background level of potent estrogens and androgens whose levels need to be evaluated in terms of potential toxicity and detrimental developmental outcomes.

Projected Research Accomplishments During Next 3 Years:
Add assays which increase the classes of hormonally active agents we can detect to include whole organism developmental assays, mammalian cell culture assays, and in-vitro receptor binding assays for estrogens, androgens, retinoic acids, arylhydrocarbons, and thyroidal compounds through collaborations with Sig Degitz (US EPA) and Caren Helbing (Univ. of Victoria, Canada) to employ DNA microarray technology to the screening of Xenopus mRNA for thyroid antagonists and agonists; 2) to expand our collaborative work with Elwood Linney (Duke Univ.) and his retinoic acid receptor based, transgenic developmental assay; 3) to explore the use of a mammalian cell culture assay for estrogenic and androgenic compounds [Ana Soto (Tufts University)] and 4) to obtain radiolabeled estradiol and testosterone, and measure the half-life of each in a composting operation and in an aqueous environmental setting and identify any metabolites and degradation products. To complete FETAX analysis of water samples; 2) to conduct FETAX assay on extracts and fractions; 3) to continue screening for BACs using the other assays described in above; 4) and to construct a soil column in the laboratory and by using radiolabeled hormones, measure the transport rate and fate of these substances in soil. Identify BACs from affected sites from fractions which show activity in the various assays described above 2) to establish screening for thyroid agonists and antagonists; 3) to obtain functional bioassays for estrogenicity and androgenicity, and 4) to complete a survey of Midwest agriculture, pristine environments, and municipal waterways.

Technology Transfer:
Our studies on determination of the presence of estrogenic compounds in water associated from MN with frog malformation using FETAX analysis and an evanescent field fluorometry based biosensor has been reported to scientists from government, industry and academia at international meetings.

PROCEEDINGS/ABSTRACTS:

Erb, J.L., Wittliff, J.L., Larsen, G.L., Magner, J., Garber, E.A.E. Determination of the presence of estrogenic compounds in water associated with frog malformations using an evanescent field fluorometry based biosensor. 2000. FASEB Journal. v. 14. A1470. Abstract #913.

Zander, A., Magner, J., Garber, E.A.E. Fetax analysis of Minnesota lake water associated with frog malformations. Great Lakes Regional Meeting: American Chemical Society. Abstract #230. p. 84

13.20

CRIS Title: The Effect of Plant Genetics and Zinc on Cadmium Concentration and Bioavailability in Crops
CRIS: 1265-42000-005
Scientists: Chaney RL
Location: Animal Manure and By-Products Laboratory, ANRI, BARC, Beltsville, MD
Contact: 301-504-8324 (P); 301-504-5048 (F); rchaney@asrr.arsusda.gov

Summary Project Aims:
In some production areas, natural soil Cd enrichment, strong soil acidity or high soil chloride promote higher uptake of Cd by crop plants to reach levels which may prevent marketing of the crop in Europe. Because excessive chronic consumption of food Cd can cause renal tubular dysfunction in humans, international standards for Cd in crops have been imposed by some nations and may be imposed by Codex. Nonoilseed sunflower kernels, durum wheat and flax grown on some soils in the Northern Great Plains have experienced market limitations in such nations, as have peanut and soybean from limited production areas. Our research helps solve this problem by identification of low Cd uptake crop genotypes which may be useful in breeding lower Cd cultivars; and by characterization of soil properties and production practices which can be used to produce crops which meet export limitation. Further, research indicates that crops differ significantly in bioavailability of crop Cd, and higher Zn in edible crop tissues can inhibit Cd absorption. A clear demonstration that crops differ in Cd bioavailability due to increased severity of Fe and Zn deficiency in subsistence crop is consumers who suffered Cd diseases from rice but not wheat or garden foods may assist the international community in developing improved regulations related to risk of Cd in foods rather than simply concentration of Cd in foods.

Summary Accomplishments During Entire Project:
The project began when sale of U.S. sunflower kernels was prevented in some countries based on Cd concentration. Our research identified soil series which could reliably produce low Cd kernels, and then identified soil properties which appear to have caused higher Cd uptake on some soil series. This knowledge gave the sunflower marketing companies the ability to purchase and market lower Cd nonoilseed sunflower kernels which protected a market for $30 million of kernels per year. Genetic testing of the U.S. germplasm bank identified higher and lower kernel Cd genotypes and the inheritance of lower kernel Cd was established. This allowed commercial breeders to use the low kernel Cd trait found in several genotypes to produce improved low Cd hybrids after moving the low Cd genes into inbreds used to make hybrids. Flax sales to Europe have essentially been prevented by German limits on Cd, and durum wheat sales are threatened by grain Cd. Corroboration that soil chloride played an important role, along with acidic soil pH, in causing higher grain Cd, allows growers to limit planting to fields which will produce a marketable crop. Also, Plant Introduction lines for flax in U.S. germplasm bank (over 3000) were grown on one soil series which has high Cd phytoavailability to allow valid comparison of genetic differences, and a wide range in grain Cd was observed (0.27 to 3.6 mg/kg dry kernels), demonstrating the potential to breed low Cd genotypes which could be marketed within market standards.

Cooperator J.J. Hammond at NDSU made crosses and grew the progenies on the field test soil to evaluate inheritance of grain Cd in flax. Cooperator J.W. Miller (ARS, Fargo, ND) had back-crossed the low Cd trait into commercially important nonoilseed sunflower inbreds for several male and several female inbreds. These were intermated in a combining ability test o evaluate how well grain Cd had been reduced in hybrids; the hybrids were grown in a field test this summer. After harvest, analysis of grain from these tests will provide the information needed to efficiently breed lower Cd sunflower kernels and flax grain.

Summary 2000 Accomplishments:
In cooperation with Dr. P.G. Reeves of the ARS-Grand Forks Human Nutrition Research Center, a feeding test was conducted to quantitate the role of crop Zn, Fe, and Ca on absorption of crop Cd by rats. Animals were fed marginal or adequate levels of these nutrients in a factorial design. The study showed that, based on kidney and liver Cd from isotope labeled sunflower kernels, kernel Fe and Ca contributed to lower bioavailability of crop Cd but Zn had less effect. This may have resulted from the high phytate levels in sunflower kernels which is well known to reduce bioavailability of food Zn.

Projected Research Accomplishments During Next 3 years:
Concentrations of Cd and other elements in the sunflower kernels and flax grain grown in a field with high soil Cd phytoavailability will be measured to assess the inheritance of grain/kernel Cd when the mature grain arrives at our laboratory. Considerable effort will be required to evaluate the data and prepare manuscripts on this overall program to identify how breeders can produce lower Cd cultivar for growers. It is expected that genetic releases will be made at the completion of this process of evaluating how well low grain Cd was attained. A new component of this research project will be initiated to characterize the role of soil, crop, and remediation treatments on Pb uptake by carrot. Carrots grown on historic orchard soils rich in Pb and As from Pb-arsenate pesticide used in apples for many decades has left high residues of Pb and As in soils of these historic orchards. As sprays on peaches and cotton similarly caused soil As to accumulate to potentially problem levels. When land use changes to vegetable production, grazing, or residential development, the high soil Pb and As may comprise risk to growers (carrot Pb exceeding FDA expectations), livestock which ingest soil while grazing, or children who ingest soil during hand-to-mouth play. We expect to characterize the relative importance of carrot cultivar, soil Pb concentration, and other soil properties; and the effect of remediation treatment with high Fe biosolids compost, or rock phosphate, on carrot Pb and As concentration both in the peel layer and in the core which is normally consumed.

Technology Transfer:
The significant differences in potential sunflower kernel Cd when crops were grown on different soil series, and different growing regions, was communicated to sunflower marketing companies associated with the National Sunflower Association and Extension agronomists in states which produce nonoilseed sunflower kernels. The important effect of high soil chloride increasing Cd uptake by sunflower and other crops was also communicated to Extension agronomists and the National Sunflower Association. High soil chloride from pedogenic sources in the Northern Great

Plains contributes substantially increases kernel Cd; that nonoilseed sunflower should not be produced on local high chloride soils was communicated to Extension advisors. Nonoilseed sunflower seed producers were given the results of genotype screening and of inheritance studies so they could develop inbreds and hybrids for commercial use. Industry representatives report that the purchase of kernels produced on specific soil series allow the industry to meet their contracts for sales of low Cd crop without breeding low Cd hybrids, but we believe that limits crop selection for growers. Cooperator Miller back-crossed the low Cd trait to male and female inbred lines commonly used in making nonoilseed sunflower hybrid cultivars so that public released inbreds and information on hybrids can provide the basis for breeding low kernel-Cd hybrids useful on all soils.

PUBLICATIONS:

None

PROCEEDINGS/ABSTRACTS:

Chaney, R.L., J.A. Ryan and J.S. Angle. Transfer of cadmium through plants to the food chain. Invited lecture at UNEP-SCOPE Workshop on "Environmental cadmium in the food chain: Sources, pathways, and risks." at the Belgian Academy of Sciences, Brussels, on 13-16 September, 2000.

CRIS Title: Identify Mechanisms of Isoflavonoid Induction in Legumes and their Phytoestrogenic Effects
CRIS: 6435-42000-013
Scientists: Cleveland TE, Boue SM, Ehrlich KC
Location: Food and Feed Safety Unit, SRRC, New Orleans, LA
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Summary Project Aims:
Hormonal changes in post-menopausal women can increase the risk of developing breast cancer, bone loss, and reduced cardiovascular health. Populations consuming a diet high in soybean phytoestrogens have lower incidences of several diseases, including breast and prostate cancer. Research has shown that certain phytoestrogens in the class of compounds known as flavonoids can be found in soybean derived foods and may reduce the risk of certain potential health problems when consumed in the diet. Identifying the phytoestrogenic activity of soybean flavonoids could ultimately benefit the nutritional health of the population in general, particularly women. Techniques are being determined by Agricultural Research Service (ARS) scientists to manipulate phytoestrogen levels in soybean seed and soy-based products to maximize their potential health benefits (and to minimize possible detrimental effects) due to their consumption. Several "elicitors" that have the ability to induce production of certain plant chemicals have been evaluated for potential use in inducing flavonoids. Collaborating scientists from Tulane University have performed a series of laboratory bioassays using various phytoestrogen compounds extracted from elicitor-treated soybean seeds. Based upon preliminary results, these inducible phytochemicals demonstrated the potential to act as phytoestrogens in animal cell based bioassays.

Summary Accomplishments during Entire Project:
This project is closely associated with the Tulane and Xavier Universities Center for Bioenvironmental Research (CBR). In the first phase of this project nearly 100 compounds in a class of chemicals known as isoflavonoids were supplied to Tulane University by ARS scientists where they were tested in animal system bioassays for phytoestrogenic activity. Several compounds showed phytoestrogenic activity in certain assays. Recent results indicate that a soybean defense chemical known as glyceollin which is induced by pest attack (and therefore defined as a phytoalexin), and other inducible defensive compounds referred to as antiestrogens inhibit the activity of estrogen. These findings have led to the isolation of other phytoalexins from different legumes for phytoestrogenic analyses, including phaseollin from green beans and pisatin from snow peas. Chemical structural features of the flavonoids were identified that are required for estrogenic and antiestrogenic activity. ARS scientists have identified several fungal-derived inducers (or elicitors) including the fungus, Aspergillus sojae, which induce phytoestrogen levels in soybean seed halves (cotyledons) several fold over that in non-induced cotyledons. Several abiotic (non-living source) elicitors have also been utilized for the induction of flavonoids, however, research has focused on the more potent elicitor systems derived from living (biotic) sources such as fungi. These experiments demonstrate precedence for our goals to: 1) manipulate phytoestrogen levels in soybean and soybean products for optimal health benefits, and 2) greatly boost phytoestrogen production to identify new trace compounds which may also have phytoestrogenic activities.

Most recently, a compound known as apigenin was discovered by ARS scientists in soybean pods. In collaborative research with the CBR at Tulane University, it was demonstrated that apigenin blocked estrogen's actions in the growth of breast cancer cells. Estrogen often is used in hormone replacement treatment in women, but it is expensive and contains chemicals that may promote proliferation of breast cancer. So, the complex estrogenic/antiestrogenic action of apigenin may be an important consideration with regards to effects on human health. Also, soybean pods, which are of little value otherwise, may be an excellent source of apigenin. IMPACT: The overall impact of this research is that the identification of beneficial flavonoids (both induced in cotyledons and/or already present without induction in cotyledons and pods) with phytoestrogenic activity could ultimately benefit human health. Technology to increase beneficial flavonoids in soybean (and other legumes) could be implemented through genetic engineering or through the use of elicitors.

Summary 2000 Accomplishments:
Research to identify the phytoestrogenic activities of certain soybean compounds called flavonoids could ultimately benefit the nutritional health of the population in general, by manipulating levels of these compounds to achieve optimum benefits in human diets. We have developed a method to induce high-level production of certain relatively understudied (with regard to human health) flavonoids, called glyceollins, in soybean seeds. The accomplishment of boosting production of these flavonoids to higher levels was key in providing sufficient quantities of the compounds for further analysis. For example, cooperators at the Tulane and Xavier Universities Center for Bioenvironmental Research, and the Tulane University Department of Chemistry showed in laboratory assays that these inducible flavonoids may have phytoestrogenic activity. IMPACT: This research could lead to a better understanding of the effects of soybean-derived flavonoids on human health and may lead to alternative or value added uses of soybean (legume) flavonoids.

Projected Research Accomplishments during Next 3 Years:
Continue to purify and characterize phytoestrogenic compounds and determine structure activity relationships in animal system bioassays in cooperation with the Tulane and Xavier Universities Center for Bioenvironmental Research and the Tulane University Department of Chemistry. Once sufficient amounts of compounds are purified and analyzed in rapid laboratory bioassays, several candidate phytoestrogens, including genistin, daidzin, malonylgenistin, malonyldaidzin, glycitein, glyceollin and apigenin, will be examined for phytoestrogenic activity in animals in cooperation with University of Arkansas. Results have indicated that soybean phytoalexins have antiestrogenic activity, therefore several phytoalexins from other legumes are being characterized. Complete laboratory experiments to elicit or induce phytoestrogens in soybean pod and seed tissues and in soybean tissue cultures using fungal and/or chemical elicitors. Also, in longer term research, the possibility of the existence of new trace isoflavonoids and flavonoids with phytoestrogenic activity will be examined in extracts of "hyperinduced" soybean. These experiments will produce information on methods to regulate flavonoid synthesis and manipulate phytoestrogen pathways in the plant.

Technology Transfer:
Over the last year several flavonoids, including apigenin and the phytoalexins glyceollin, phaseollin, and pisatin were delivered to Tulane University and Tulane's Center for Bioenvironmental Research for testing in animal bioassays for phytoestrogenic activity. Also, research is in progress to develop a value-added product rich in beneficial soy isoflavones. However, many obstacles have to be overcome before a viable food product is produced. Potential constraints: 1) The degree of industry willingness to adopt new production/processing methods to enhance isoflavone content of existing products, 2) availability of germplasm if induction methods are optimal only by using specific soybean varieties or types, 3) public willingness to accept/consume products that may be enhanced as a result of chemical application or exposure to abiotic elicitors inducing greater amounts of flavonoid compounds in seed, when evidence shows that these compounds may be responsible for disagreeable flavor qualities in many soybean products.

PUBLICATIONS:

Boue, S.M., Carter, C.H., Ehrlich, K.C., Cleveland, T.E. Induction of the soybean phytoalexins coumestrol and glyceollin by Aspergillus. Journal of Agricultural and Food Chemistry. 2000. v. 48(6). p. 2167-2172.

PROCEEDINGS/ABSTRACTS:

Boue, S.M., Weise, T.E., Carter-Wientjes, C.H., Cleveland, T.E. Estrogenic and Antiestrogenic Activity of Legumes and Other Plants Containing Phytoestrogens. 52nd Southeast/56th Southwest Combined Regional Meeting, December 6-8, 2000, New Orleans, LA, and Tulane CBR hormone Meeting, October 15-18, 2000, New Orleans, LA.